THE EFFECTS OF OIL AND DISPERSED OIL ON THREE TEMPERATE AUSTRALIAN SEAGRASSES – SCALING OF POLLUTION IMPACTS

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Certificate of Authorship

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Abstract

The thesis is a comprehensive assessment of the effects of oil and dispersed oil on subtidal seagrass using a range of *in situ* and laboratory experiments on whole plants and seagrass leafblade sections. Apart from assessing the effects of oil and dispersed oil on seagrass between seasons, locations, and morphologically different species, the study determines whether laboratory results are indicative of those obtained *in situ* as an initial step in developing a rapid laboratory testing protocol for seagrass assessment. Petrochemical treatments, consisting of a range of concentrations of the water accommodated fraction (WAF) of oil alone (Tapis crude, IFO-380), dispersant alone (Corexit 9527, Ardox, Slickgone, Corexit 9500) and dispersed oil were exposed to whole plants, in both the laboratory and *in situ*, for ten hours followed by a four day recovery period, and for five hours in the leafblade experiments. Photosynthetic health was monitored by assessing the effective quantum yield of photosystem II ($\Delta F/F_m'$) and chlorophyll *a* pigment concentrations, whilst semi-quantitative methods of total petroleum hydrocarbon (TPH) concentration were used to determine the percent TPH remaining in the water column following the exposure period.

In most cases, the non-dispersed oils, Tapis crude oil and IFO-380, had less of an impact to both *Zostera capricorni* and *Halophila ovalis* than the dispersed oil treatments, whilst *Zostera muelleri* did not show any negative impact from either dispersed or non-dispersed Tapis crude oil. Winter *in situ* experiments found slightly greater reductions of $\Delta F/F_m'$ in *Z. capricorni* in most treatments compared with summer *in situ*, but generally there was minimal impact whilst *Z. muelleri* exhibited a stimulatory response to both non-dispersed and dispersed Tapis crude oil in Corio Bay, Victoria (summer *in situ* only). Laboratory whole plant experiments found *Z. capricorni* was for the most part less resilient to Tapis crude oil (non-dispersed and dispersed) treatments than *Halophila ovalis* whereas, with exposure to IFO-380 (non-dispersed and dispersed) *H. ovalis* was less resilient than *Z. capricorni*. Quite severe, and, or prolonged, photosynthetic stress was evident in both *Z. capricorni* and *H. ovalis* when exposed to most of the dispersant alone treatments (Corexit 9527, Ardrox and Corexit

9500), however the Slickgone alone treatment caused only a very short-lived stress response in *H. ovalis* only. The results of the laboratory whole plant experiments, conducted under Sydney summer water temperature conditions, were generally more similar to those observed in the summer *in situ* experiments than those observed in winter *in situ*. The effects to the leafblades of *Z. capricorni* were commonly greater than those observed in the whole plant experiments, even within the short exposure period. $\Delta F/F_m'$ appeared a more reliable indicator than that achieved with the chlorophyll *a* pigment analyses. Large differences in the percent TPH recovered between *in situ* and laboratory experiments suggests microbrial activity and sediments play a substantial role in the partitioning of oils in these experiments. This research suggests that assessments of seagrass health in laboratory experiments can in some cases be representative of that observed *in situ* when similar experimental conditions are maintained. The increased sensitivity of leafblade sections is considered beneficial when rapid comparisons of different petrochemical impacts to seagrass are required, i.e. once an oil spill has occurred.

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List of Abbreviations

AMSA	Australian Maritime Safety Authority
ANOVA	Analysis of Variance
API	American Petroleum Industry
bbl	barrel
BTEX	benzene, toluene, ethylbenzene, xylene
Chl *a*	Chlorophyll *a*
cSt	centistoke
$\Delta F/F_m'$	Effective quantum yield of photosystem II
EWG	Environmental Working Group
GC-MS	Gas Chromatography –Mass Spectrometry
HPLC	High Performance Liquid Chromatography
mg L^{-1}	milligrams per Litre
NEBA	Net Environmental Benefit Analysis
NOAA	National Oceanic and Atmospheric Association...
OSC	Oil Spill Coordinator
PAH	Polycyclic Aromatic Hydrocarbon
PAM	Pulse Amplitude Modulation
ppm	parts per million
ppt	parts per thousand
PSI	Photosystem I
PSII	Photosystem II
psu	percent salinity unit
rmANOVA	repeated measures Analysis of Variance
TPH	Total Petroleum Hydrocarbons
UV	Ultra-Violet
UVF	Ultra-Violet Fluorescence
WAF	Water Accommodated Fraction
WSF	Water Soluble Fraction
µg L^{-1}	micrograms per Litre

1 Introduction

The effects on subtidal seagrass from the application of dispersants to oil spills remain unclear (AMSA 2008). The Environmental Working Group of the National Plan to Combat Marine Pollution has defined this lack of knowledge as being of concern. To disperse or not to disperse an oil spill is usually a "trade-off" between the relative importance of subtidal resources and shoreline habitats (Fingas 2001). Dispersant application is often sought when sensitive shoreline resources (eg. mangrove habitats, nesting bird colonies) are at a clear risk of oil contamination if the oil were to be left to degrade and weather naturally. Chemically dispersing the oil into the water column may therefore be justified to decrease the risk of the oil coming closer to shore. In dispersing the oil spill, however, the hydrocarbon concentration in the water column is greatly increased and thus increases the potential risk to subtidal organisms, such as seagrasses (Fingas 2001; NRC 2005; AMSA 2008).

Declines in seagrass habitats have been reported worldwide as a result of natural events and human-induced stress (Larkum & West 1987; Kirkman 1997; Short & Wylie – Echevaria 2000; Green & Short 2003). Seagrass meadows are extremely productive environments; they uptake nutrients from surrounding waters, stabilise the sediment and provide habitat and shelter for many species including those of commercial and recreational importance (Kirkman 1997; Hemminga & Duarte 2000; Mateo *et al.* 2006). Any controllable anthropogenic pressures need to be prevented or minimised where possible to reduce further degradation of these critical habitats.

To reduce further pressure to seagrass meadows, an improved understanding of the effects of oil spills, and dispersants used in mitigation procedures, is required (AMSA 2008). While studies have investigated the effects of petrochemicals on seagrasses (e.g. Thorhaug *et al.* 1986; Macinnis & Ralph 2003a); there has been a variety of, and often conflicting, findings from this research. A more comprehensive approach, incorporating

methods, and utlising the findings, of previous research, may provide better assistance to the oil spill mitigation decision making process.

1.1 Seagrass

Seagrass are true marine angiosperms living fully submerged in seawater and are found throughout the world in coastal and estuarine zones (den Hartog 1970). Seagrasses inhabit tropical, temperate and even Arctic environments and species are present in waters around every continent except Antarctica. Worldwide seagrass coverage is difficult to measure, but has been estimated at about 0.6×10^6 km^2 (Charpy-Roubad & Sournia 1990). In 1997, the estimated seagrass coverage in Australia was 51, 000 km^2 (Kirkman 1997) with Western Australia and the Torres Strait having the greatest percentage contribution at 43.2 % (2, 200 km^2) and 35.3 % (1, 800 km^2) of the total area, respectively (Kirkman 1997).

Seagrasses are generally classified into two families, encompassing 12 genera containing about 50 species (Hemminga & Duarte 2000). Australian seagrass show a high degree of endemism and there are representatives from 12 genera (Kirkman 1997; Butler & Jernakoff 1999). Australian seagrass are generally divided into tropical and temperate species with the geographical divide considered as Moreton Bay on the east coast and between Perth and Shark Bay on the west coast (Kirkman 1997). Until recently it was considered there were 18 species inhabiting tropical environs and 12 inhabiting temperate environs in Australian waters (Short *et al.* 2001). However, research during the last decade suggests the merging of several 'species' into one (Les *et al.* 2002). Developments in molecular and morphological identification techniques have led to a revised classification of several 'species' to subspecies levels (eg. *Zostera muelleri* and *Zostera capricorni* are now termed as subspecies of *Zostera muelleri*) reflecting a lack of morphological and molecular distinction between certain species (Les *et al.* 2002; Jacobs *et al.* 2006).

1.1.1 Seagrass Biology

General Structure of the plant

The seagrass plant is divided into above ground blades and below ground rhizomes and roots. The above ground biomass consists of leaves (blades) and leaf sheaths. In most species, new shoots are developed from the apical meristem and are protected by older sheaths surrounding new blades (Kuo & McComb 1989). Leaf morphology varies between species, but generally the blades are long, slender and strap-like (eg. *Zostera* spp.) or broad and paddle-shaped (eg. *Halophila* spp.). The blades of the seagrass lack stomata; instead the surface is covered by a thin porous or perforated cuticle across which the transfer of gases and solutes takes place (Hemminga & Duarte 2000). The epidermis is rich in chloroplasts being the main site of photosynthesis in the plant (Kuo & McComb 1989).

The below-ground structure of the seagrass comprises the roots and rhizomes. It is the vast interconnected mats of rhizomes underneath the sediment that forms the structural foundation of the seagrass meadow. The roots and rhizomes act to anchor the plant, store carbohydrates, and absorb nutrients (Hemminga & Duarte 2000). This below-ground structure accounts for about 50 to 75 % of the total biomass of the plant (Zieman *et al.* 1984).

Photosynthesis

Like all phototrophic organisms, seagrass utilise energy from sunlight for growth via photosynthesis. Thylakoid membranes within the chloroplast contain the Photosystem I (PSI) and II (PSII) reaction centres, protein complexes involved in photosynthesis in the plant (Touchette & Burkholder 2000; Larkum *et al.* 2006). Photosytems I and II utilise photon absorption by the chlorophyll pigments within these centres for the conversion to energy usable to the plant. The pigment composition in seagrasses is similar to that in most angiosperms, containing chlorophylls *a*, *b* and carotenoids (Beer 1998).

Many recent studies of photosynthesis in seagrasses have examined photochemical processes at PS II using Pulse Amplitude Modulated (PAM) fluorometric techniques (eg. Beer & Bjork 2000; Seddon & Cheshire 2001; Enriquez *et al.* 2002; Ralph *et al.* 2002; Schwarz 2004). As plants photosynthesise, the energy captured can be dissipated in three ways; as photochemical quenching to ultimately fix carbon; dissipated as heat, non-photochemical quenching; or re-emitted as fluorescence (Maxwell & Johnson 2000). It is the measure of these three parameters that form the basic principle of the chlorophyll *a* fluorescence technique and the popularity of the technique is due to its capacity to determine rapid non-destructive measurements that can also be performed *in situ* (Schreiber *et al.* 1994; Macinnis & Ralph 2003a, b).

Photosynthesis in seagrass is relatively well–studied, but the geographical expanse of their habitat and the associated morphological differences leads to variations in photosynthetic estimates (eg. photosynthetic rates and optimal requirements) (Hemminga & Duarte 2000). Seasonal differences in species growth rates are also apparent. Seasonal variation of water temperature coupled with increased light attenuation and photoperiod in summer than winter may interact to determine the growth rate of the plant (Masini & Manning 1997; Bostrom *et al.*. 2004). In temperate *Zostera capricorni*, greater shoot density, leaf length and above–ground biomass have been recorded in summer compared with winter (Harris *et al.* 1980; Kirkman *et al.* 1982, Larkum *et al.* 1984).

Reproduction

Reproduction in seagrass generally occurs via sexual means (Hemminga & Duarte 2000), however, seagrass plants do not flower regularly and carbon allocation invested in flowering is low (Hemminga & Duarte 2000). Timing of flowering varies latitudinally, but for temperate species flowering typically occurs in late spring coinciding with increased irradiance and water temperature (Phillips *et al.* 1983). Some species may only flower once in the lifetime of the particular plant and seed dispersal is often within close proximity to the plant (Orth *et al.* 1994). Often following recovery

from a stress event, seagrass will recolonise via horizontal extension of the rhizomes rather than through sexual means. Rasheed (1999) created small (0.25 m^2) gaps in *Z. capricorni* meadows to simulate those commonly arising from anchoring and propeller damage. He found that recolonisation occurred almost exclusively via extension of surrounding rhizomes with no significant recovery by sexual means (Rasheed 1999).

1.1.2 Habitat Requirements

Seagrasses inhabit a range of environments. Some seagrass in Western Australia can withstand high energy environments, but those occurring along the south-eastern coastline of Australia are limited to protected areas where flow rates and wave action are reduced (Leadbitter 1986; Kirkman 1997). They are thus constrained and found commonly in estuaries, sheltered bays and on the lee side of islands and coral reefs. Seagrass habitat is defined by their need for sunlight (Duarte 1991), nutrients (Perez *et al.* 2007) and a suitable substrate for anchorage (Hemminga & Duarte 2000). The salinity ranges of seagrasses vary, but seagrasses generally occur in full seawater, 33 % percent salinity unit (psu) (Larkum *et al.* 1989).

The level and attenuation of light is a critical determinant of seagrass distribution and depth limits are largely determined by this. Seagrass have high minimal light requirements; generally about 11 % of surface irradiance (Duarte 1991; Ralph *et al.* 2007). Seagrasses are highly sensitive to periods of low light, whilst high levels of light can lead to photoinhibition (Dawson & Dennison 1996; Ralph *et al.* 2007). Although *Halophila capricorni* has been found at depths of about 90 m, the majority of seagrasses grow at depths no greater than 25 m (Duarte 1991). Light attenuation is affected by the turbidity of the water which again determines the habitat of seagrass. Where the water is highly turbid, seagrass growth is likely to occur at shallower depths to account for the decrease in light attenuation. Indications of light stress in seagrass include decreases in below-ground biomass, reduction of the chlorophyll *a* content of leaves and impacts to the photosystems (Coles & McKenzie 2004; Ralph *et al.* 2007).

Seagrasses also require inorganic carbon for growth which is taken up at the leaf surface, so the habitat must also provide this. Nutrient availability, chiefly nitrogen and phosphorous, is required for growth of the plant. However, high nutrient levels can become excessive and negatively impact the plant (Perez *et al.* 2007) and consequently seagrasses are unable to grow in areas of high organic content.

1.1.3 Value of Seagrass

Seagrasses provide a range of services to the habitat; they stabilise sediment through their anchoring root system; remove sediment and nutrients from coastal waters; and provide shelter and habitat for fish and invertebrates (Kuo 1982; Bell & Westoby 1986; Duarte & Chiscano 1999; Hemminga & Duarte 2000). Seagrass are major global primary producers, ranking amongst the most productive ecological systems (Cambridge & Hocking 1997; Hemminga & Duarte 2000; Mateo *et al.* 2006). Whilst difficult to calculate, the average net primary biomass productivity of seagrass meadows has been estimated at about 1012 g DW m^{-2} y^{-1}(Duarte & Chiscano 1999). Compared with the estimated productivity of coral reefs, 292 g DW m^{-2} y^{-1} (Crossland *et al.* 1991), seagrass productivity is high. The ecosystem services they provide have been estimated at greater than US$3.8 trillion annually (Costanza *et al.* 1997). The relative value of seagrass meadows can be defined by a comparison of the ecosystem services provided by forests (US$4.7 trillion) and coral reefs (US$0.4 trillion) (Costanza *et al.* 1997).

Stabilisation of sediment is one of the major values of seagrass meadows. The vast interconnected mats formed by the rhizomes and roots bind sediment and act to stabilise the immediate environment (Hemminga & Duarte 2000). Even when the above ground biomass has been removed, the underlying rhizomes can still act to stabilise the environment. This sediment stabilisation reduces the capabilities of erosion thereby enabling habitat for other species.

Seagrass play an important role in the marine carbon cycle with estimates of about 15 % of the total carbon storage in marine ecosystems (Hemming & Duarte 2000). Seagrass meadows act as both a source and a sink for nutrients in the environment. Decaying material from within the meadow acts as a source of nutrients, whereas the seagrass plant itself actively uptakes nutrients, such as nitrogen and phosphorous, from the surrounding water and sediments (Short & Short 1984; Perez *et al.* 2007). The reduction in current velocity by the canopy enables suspended particles to settle out of the flowing water (Fonesca *et al.* 1983; Perez *et al.* 2007). These particles are commonly rich in nutrients that become incorporated into the sediment, and or, are taken up by the seagrass plant.

In many estuarine systems, seagrass meadows form the basis of the trophic food web. Dead seagrass blades are broken down by microbial activity, and invertebrates, such as crabs, actively feed on the detritus (Klump *et al.* 1989). Although few species, especially in temperate environments, actively feed on the seagrass plant, many species feed on the epiphytic organisms attached to the seagrass blade (Klump *et al.* 1989). Provision of habitat for other species is considered one of the major values of seagrass meadows. The stabilisation of habitat; protection from predators; shelter; and the abundance of food within seagrass meadows provides for ideal conditions for many species (Middleton *et al.* 1984; Poiner *et al.* 1993; Jenkins & Wheatley 1998). Many recreational and commercial fished species inhabit seagrass meadows with many juvenile fish and invertebrates utilising the systems as nursery grounds (Jenkins & Wheatley 1998). Middleton *et al.* (1984) reported that 50 % of the dominant fish species associated with *Z. capricorni* and *Posidonia australis* meadows in Botany Bay, New South Wales, Australia, were of economic importance. Further, other plants and algae prosper in seagrass meadows due to the extension of habitat (Klump *et al.* 1989). When the seagrass meadow is reduced or lost, many species associated with the meadow are also affected.

1.1.4 Seagrass Declines

Decline of seagrass meadows have been reported worldwide and most studied have shown some level of decline (Short & Wyllie-Echeverria 1996). Reports of loss have been attributed to both natural and anthropogenic events. Natural events which cause seagrass loss include severe weather events, progressional change in the ecosystem, natural fluctuations and increases in temperature, and natural cyclic fluctuations within the seagrass community (Larkum & West 1983; Kirkman 1997; Chollett *et al.* 2007). Seagrass decline has been documented following major storms and cyclones. For example, Poiner *et al.* (1989) reported the loss of 150 km^2 area of seagrass in the coastal area of north - Western Australia following a major cyclone. Kirkman (1997) reported a loss of Australian seagrass meadows of over 450 km^2 due to anthropogenic reasons and another 1, 000 km^2 lost through natural events, such as floods and cyclones (Kirkman 1997). However, in Hervey Bay (Queensland) alone, approximately 1, 000 km^2 of seagrass was lost following floods and a cyclone in 1992 (Preen *et al.* 1995). This loss represented one of the greatest reported losses of seagrass and coincided with the death of an "unprecedented number" of dugongs, likely from starvation (Preen & Marsh 1995; Preen *et al.* 1995). Anthropogenic reasons for decline in seagrass distribution include pollution induced stress from heavy metals, petrochemicals and herbicides; dredging-induced erosion, turbidity and alterations to wave regime; eutrophication from sewage input and nutrient runoff, and introduction of invasive species (Larkum & West 1990; Kirkman 1997; Prange & Dennison 2000; Occhipinti-Ambrogi & Savini 2003; Bernard *et al.* 2007).

1.1.5 Threats to Seagrass

Pollution-induced stress to seagrass occurs as a result of oil spills (discussed in more detail later in this chapter), but also through the leaching or introduction of heavy metals and herbicides into the environment. One of the key modes of action of pollutants is the inhibition of photosystem II (PSII) supported by a compelling body of literature from

exposure to herbicides (Scarlett *et al.* 1999b; Haynes *et al.* 2000; Ralph 2000; Macinnis & Ralph 2003b; Macinnis-Ng & Ralph 2004); metals (Ralph & Burchett 1998b; Prange & Dennison 2000; Macinnis & Ralph 2004); and petrochemicals (Ralph and Burchett 1998a; Macinnis & Ralph 2003a). Herbicide contamination has been shown to occur in nearshore environments (Haynes *et al.* 2000). Haynes *et al.* (2000) investigated the impacts of the herbicide diuron to tropical seagrass from the Great Barrier Reef and found a decrease in photosynthetic efficiency, linked to an effect at photosystem II. Heavy metal accumulation in the sediments of industrial ports and harbours is also a concern for the health of seagrass meadows as the re-suspension of particles can occur following dredging activities and storm events. Other contaminants which induce stress in seagrasses include antifouling agents formerly used in shipping. The herbicide Irgarol 1051, previously used as an antifouling agent, was shown to decrease photosynthetic rates of *Zostera marina* (Scarlett *et al.* 1999b) and high concentrations of the antifouling agent Irgarol 1051 (up to 118 ng g^{-1}) were detected in seagrass samples collected from Queensland coastal waters (Scarlett *et al.* 1999a).

Global warming is further considered to have effects on seagrasses via sea level rise and shifts in invasive species (Bjork et al. 2000). Increased turbidity is also projected to occur in areas where the intensity and frequency of severe weather events are projected to occur (Bjork et al. 2000). An increase in sea temperature and UV irradiance may lead to increased rates of respiration and photoinhibition of seagrasses (Masini *et al.* 1995; Touchette & Burkholder 2000).The increase in the sea surface temperature may also negatively impact seagrass through decreased light levels from the increased potential of epiphytic algal growth (Bjork *et al.* 2008). Furthermore, as the water quality is tightly interconnected to the health of the seagrass, any changes to the pH, O_2 and CO_2 in the water, are likely to impact the photosynthetic output of the plants (Hemminga & Duarte 2000; Bjork *et al.* 2008).

Invasive species pose a threat to the distribution, abundance and density of seagrass meadows. Research has shown that when a seagrass meadow is already under some pressure, such as by eutrophication, opportunistic species can be more successful at

colonising the habitat. Infestations of the invasive plant *Caulerpa taxifolia* have been reported amongst seagrass meadows in New South Wales (Glasby *et al.* 2005). *Caulerpa taxifolia* has overtaken large areas of *Posidonia oceanica* meadows in the Mediterranean (Occhipinti-Ambrogi & Savini 2003) and appears more successful in areas where the seagrasses were already degraded due to other stressors, including eutrophication.

Eutrophication has been described as probably the greatest cause of seagrass decline worldwide (Hemminga & Duarte 2000). Eutrophication can arise through natural events, however, there is substantial evidence associating seagrass decline with industrial development (Neverauskas 1987; Kirkman 1997; Hemminga & Duarte 2000). Increase in nutrients in the water column increases the growth of epiphytic algae on the seagrass blades, thus reducing sunlight reaching the plant, leading to a decrease in photosynthesis (Shepherd *et al.* 1989; Kirkman 1997). Neverauskas (1987) reported the complete loss of a 365 ha (3.65 km^2) seagrass meadow and an affected area of 1900 ha (19 km^2) within four years of commencement of a sewage treatment plant in South Australia. The loss was attributed to increased nutrients and associated increase in epiphytic growth from the digested sludge of the sewage outfall. Some eight years following the decommissioning of the sewage treatment plant, Bryars and Neverauskas (2004) found some recovery had occurred but, suggested that "many decades" were required for the meadow to return to its former state.

1.1.6 Recovery of Seagrass

Recovery of seagrass will be determined by the nature, the magnitude and the duration of the disturbance (Zieman *et al.* 1984). Recovery of seagrass is species specific with variations occurring between the size and growth rates of large, slow-growing species (eg. *Thalassia, Posidonia*) compared with small fast-growing species (*Halophila* spp) (Cheshire *et al.* 2002; Bryars & Neverauskas 2004). Larger, slower growing species have been shown to endure unfavourable conditions for the longer period of time;

however, if the seagrass is lost due to long-term or serious impacts, then these species have been shown to have little if any recovery and limited recolonisation (Cheshire *et al.* 2002). Alternatively, small fast-growing species tend to be impacted at greater rates than the larger species, but are able to recolonise at faster rates (Cheshire *et al.* 2002; Bryars & Neverauskas 2004). Bryars & Neverauskas (2004) described *H. australis* as an "opportunistic coloniser" when noting its rapid colonisation over areas once inhabited by large slow-growing *Posidonia oceanica*. Similarly, Larkum and West (1990) found areas of seabed within Botany Bay once inhabited by *Posidonia australis* had been recolonised by *Z. capricorni*.

Research suggests that the species composition within a seagrass meadow is likely to be altered following determinantal impacts, and that the time required for the meadow to return to its former state could be "many decades" (Bryars & Neverauskas 2004); up to and beyond 60 years (Kirkman & Kuo 1990) or indefinite (Zieman *et al.* 1984). Zieman *et al.* (1984) attempted to synthesise the level of effect on the seagrass ecosystem and the associated recovery times following a disturbance, shown in Table 1.1.

Recovery times ranged from weeks, whereby only faunal damage within the community was evident, to an unknown, possibly indefinite, recovery time following severe damage to the system (Zieman *et al.* 1984). The authors suggested the following management procedures based on the level of disturbance (Table 1.1) to the seagrass communities:

> Following disturbance to a seagrass system, the primary management strategy and objectives should be directed toward minimizing the impact. While all efforts should be directed toward keeping damage at Level 1 or 2, where recovery may proceed naturally, it is imperative to keep Level 3 damage from becoming Level 4, where recovery or restoration to a functioning seagrass system is not possible. (Zieman *et al.* 1984, p. 54).

Whilst seagrass meadows have been shown to recover following stress, the time taken to recover, coupled with shifts in community composition, clearly highlight the need for

a reduction in further stress to these critical habitats. The management strategy chosen to mitigate an oil spill occurring within the vicinity of a seagrass habitat may be one such instance whereby a reduction in further impacts from the anthropogenic stressor may be achieved. A variety of procedures are commonly used to mitigate an oil spill but limited knowledge about the impacts some of these, such as the application of chemical dispersants, might have on seagrass, can hinder the mitigation response. If viable attempts are to be made to reduce further stress to these systems, then an increased understanding of these oil spill mitigation procedures on seagrass health is required.

Table 1.1: Damage levels and recovery of seagrass ecosystems following disturbance (Adapted from Zieman *et al.* 1984).

Damage level	Plant Effects	Associated Community Effects	System Fate	Recovery Time
1	No visible damage	Possible faunal damage	Natural recovery	Weeks to years
2	Leaf damage and removal	Faunal damage may be extensive	Natural recovery likely	6 months to years
3	Severe damage to rhizomes	Faunal damage is likely extensive	Natural recovery slow or unlikely	5 years to decades
4	Severe system damage	System completely altered	Return to same state not possible	?

1.2 Oil

Australia has the fifth largest shipping industry in the world by volume and kilometres travelled (Gilbert *et al.* 2003), couple this with major transport routes hugging the coastline; near-shore environments, including seagrass meadows, are continually at risk from oil spills (Lipscombe 2000; Nelson 2000). The Montara Wellhead (WA) leak,

beginning in August 2009, was one of the largest oil spill incidents in Australian history. Whilst an accurate spill size could not be determined due to the nature of the leak, initial estimates by PTTEP Australasia (the company operating the Oil Platform) were that 64 tonnes of oil per day was being lost; and the leak continued for more than ten weeks (AMSA 2009). Fortunately, in terms of seagrass meadows at least, an independent report (Mustoe 2009) suggested that subtidal seagrass meadows were of "limited vulnerability" due to their distance from the oil platform, for example Ashmore Reef seagrass meadows were some 150 km away. Table 1.2 lists some of the major shipping-related oil spills recorded in Australian waters. The loss of 270 tonnes of heavy fuel oil from the *Pacific Adventurer* near the coast of Cape Moreton (QLD) in 2009; the breaching of the hull of the *Global Peace* (2006) in Port Curtis, Gladstone Harbour (QLD); and the spill of 95 tonnes of light crude oil from the *World Encouragement* in Botany Bay (1979) all occurred within close proximity to subtidal seagrass meadows. In the case of Botany Bay and Cape Moreton, at least part of those seagrass meadows were, or now are, encompassed within RAMSAR wetlands of international significance (DEWHA 2010), highlighting both the extreme importance of the seagrass and the risk posed to these areas. Clearly subtidal seagrass inhabit areas where oil spills commonly occur, in nearshore and inshore environments. However, oil spills are complex events, and as such, a variety of factors interact to determine the conseqences of such events.

The variety of effects resulting from those spills previously mentioned, and from other spills, is obviously dependent on the volume of oil, the environment into which the oil is spilt and the weather conditions at the time of the spill. Another primary factor in determining the impact of the spill event, is the type of oil spilt. Two major types of oil commonly spilt are crude and heavy fuel oils. Spills of crude oil generally result from the loss of crude oil cargo on tankers or accidents whilst berthing (for example, *Laura D'Amato* tanker, Sydney, 1999; *World Encouragement* tanker 1979; Table 1.1) (Page *et al.* 2000) while heavy fuel oils (or bunker oils) are commonly spilt following damage to the hull (eg. *Kirki*, WA, 1991). As it is the chemical composition of oil that governs its

physical behaviour, knowledge of the composition of these oils is necessary to determine how they may react in the in the marine environment.

Table 1.2: Oil spill incidents in Australian waters ($^{+}$ indicates at least the stated volume was spilt). (adapted from Nelson 2000; www.amsa.gov.au)

Volume (t)	Oil Type	Location	Vessel	Year
17,280	Light crude	West Australia (WA)	*Kirki*	1991
325 $^{+}$	Bunker fuel	Hebes Reef (TAS)	*Iron Baron*	1995
300 $^{+}$	Bunker fuel	Port Bonython (SA)	*Era*	1992
270 $^{+}$	Heavy fuel	Cape Moreton (QLD)	*Pacific Adventurer*	2009
250 $^{+}$	Light crude	Sydney Harbour (NSW)	*Laura D'Amato*	1999
95	Crude	Botany Bay (NSW)	*World Encouragement*	1979
25	Heavy fuel	Gladstone Harbour (QLD)	*Global Peace*	2006

1.2.1 Crude oil

Crude oil is the raw oil product that is extracted from the seabed or other underground reserve. It is a naturally occurring product with seeps occurring on a daily basis in many parts of the oceans (Kvenvolden & Cooper 2003), including in Australian waters (Burns *et al.* 2010). Natural seeps of oils cause no concern to the biological community, however human–induced oil spills which result in much larger volumes of oil being released in an area at a particular time do. Oil concentrations above that which the environment is capable of breaking down makes oil spills a potential risk to biological resources in the vicinity of a spill.

Crude oils are complex mixtures comprising literally, thousands of components (Wang & Fingas 1995; Clark 2002). The chemical composition of oil can vary between different regions, and to a lesser extent between oil fields within the same region, within

the same oil field at different times and within the same oil field when extracted at different depths (Neff 1990; NRC 2003; Nemr 2006). Although each oil sample is therefore unique, general classifications can be made based on characteristic chemical components. Hydrocarbons account for about 97 % of most oils but oils also comprise minor elements of nitrogen, oxygen and sulphur, accounting for about 3 %, and trace amounts of other elements such as vanadium, nickel iron, sodium, copper and uranium (UNEP 1992; Gilfillan 1993; Nemr 2006; NRC 2003). The hydrocarbons within oils can be further categorised into saturates, aromatics, olefins and polar compounds based on their chemical structure (UNEP 1992; Gilfillan 1993; API 1999).

Aromatic hydrocarbons are commonly considered the most acutely toxic fraction to organisms as many are soluble in seawater (Singh & Gaur 1990; Holliger & Zehnder 1996; Clark 2002). The water accommodated fraction (WAF), previously referred to as the water soluble fraction (WSF) by some authors, is the fraction that has the potential to contact and impact organisms (Saeed & Al-Mutairi 1999; Singer *et al.* 2000). Aromatic hydrocarbons represented about 90 % of the water accommodated fraction (WAF) of crude oils in a study by Carls and Rice (1990). Monocyclic aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes (BTEX) are commonly the major component of the WAF, but polycyclic aromatic compounds are also present (Carls & Rice 1990; Holliger & Zehnder 1996; API 1999).

Low molecular weight compounds such as benzene are highly volatile and can be lost quite rapidly compared with high molecular weight compounds. Generally, crude oils are comprised of a high proportion of highly volatile, light weight components, and as such a large portion of the toxic components are lost quite rapidly (Wang & Fingas 1995; Lee & Page 1997; Wang *et al.* 1998). Table 1.3 shows the different volatilities and resultant exposure risk to organisms of the different molecular weight compounds of oil. The toxicity of crude oil is often a function of the exposure time of the oil in the environment, and when it comes into contact, or within the surrounds of a biological resource (Fuller & Bonner 2001; Clark 2002).

1.2.2 Fuel oil

Crude oil is refined into other products including for fuel used in shipping. During the refining process lower molecular weight compounds are removed. As such, fuel oils (or bunker oils) have a larger proportion of high molecular weight compounds, typically C_{20} through to C_{50} (Table 1.3) than crude oils (Ansell *et al.* 2001; Clark 2002; Hatlen *et al.* 2010).

Table 1.3: Characteristics and examples of the different molecular weight components within crude oil (adapted from API 1999).

Molecular Weight (no. of carbon atoms)	Volatility	Exposure risk	Example
Low (1 to 10)	High	High acute toxicity, rapid evaporation, dissolution	Benzene
Medium (11 to 22)	Medium	Medium – high acute toxicity, less evaporation than above	phenanthrene
High ($\geq$ 23)	Low	Long residence time, toxicity due to persistence in environment	Asphaltenes

Fuel oils are highly viscous, comprising the tarry residues of crude oil and complex mixtures of aliphatics, aromatic compounds, bitumens and asphaltenes (CONCAWE 1998; Ansell *et al.* 2001; Hatlen *et al.* 2010). Due to their high viscosity, heavy fuel oils are far more persistent in the marine environment compared with most of the crude oils (Ansell *et al.* 2001; Lunel & Lewis 2001). Lee *et al.* (2003), found traces of bunker oil in intertidal sediments thirty years after an oil spill, following the grounding of a tanker. Although fuel oils are considered less toxic to organisms than crude oils, the increased persistence of heavy fuel oils in the marine environment increases the potential risk of shoreline stranding of the oil, and of the oil coming into contact with organisms (Ansell *et al.* 2001; Lunel & Lewis 2001).

1.2.3 Weathering of Spilt Oils

Once oil is spilled into the marine environment, it begins to weather by being subjected to physical, chemical and biological processes which act to alter its physical and chemical composition (Ali *et al.* 1995; Clark 2002; Ziolli & Jardim 2002). Wave strength, tidal flow, and ultraviolet (UV) radiation all act to break up an oil slick whereas sea temperature, air temperature and the salinity of the water affect the physical and chemical properties of the oil (Saeed & Al-Mutairi 2000; Maki *et al.* 2001; Al-Lihaibi 2003). Furthermore, UV radiation not only has the potential to break down oil slicks, but also to increase the toxicity of certain components within the oil. For example the photo-oxidation of polycyclic aromatic hydrocarbons (PAHs) with exposure to UV light can greatly increase their toxicity (Maki *et al.* 2001; Lee 2003). Figure 1.1 shows the processes that act upon an oil once it has been spilt into the marine environment and Figure 1.2 shows the times associated with these weathering events.

Within the first 24 hours of a spill, evaporation acts to remove a large portion of the low-weight aromatics hydrocarbons whilst spreading, dissolution and dispersion act to dilute the oil in the environment (Figure 1.1). Within 24 hours of the *Lara D'Amato* spill in Sydney Harbour (1999), it was estimated that as much as 50 % of the oil had been lost largely due to evaporation (Lipscombe 2000). Other processes such as sedimentation and biodegradation, may occur after a longer period of time, but still play a major role in the weathering and breakdown of oil in the environment (Figure 1.1).

1.3 Oil Spill Mitigation

Several methodologies to clean up an oil spill are currently used in Australia and in other countries (Lipsombe 2000; Chapman *et al.* 2007). These include leaving the oil to breakdown naturally; containing the oil spill with booms or other containment devices; using 'skimmers' to suck the oil and water mixture onto a vessel; bioremediation techniques to increase the bacterial breakdown of the oil; and chemically dispersing the oil into the water column. In many cases, attempting to contain and recover the oil is logistically impossible, for instance during severe weather events or at an isolated

location which requires lengthy transport of containment gear (NRC 2005; AMSA 2007).

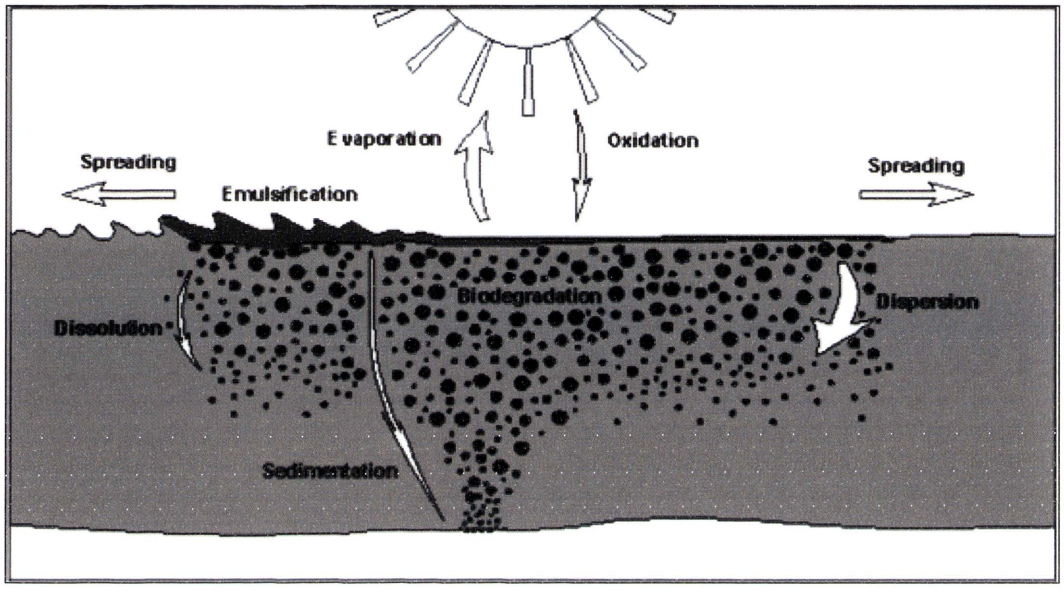

Figure 1.1: Schematic diagram of the weathering processes and fate of spilled oil in the marine environment (www.itopf.com).

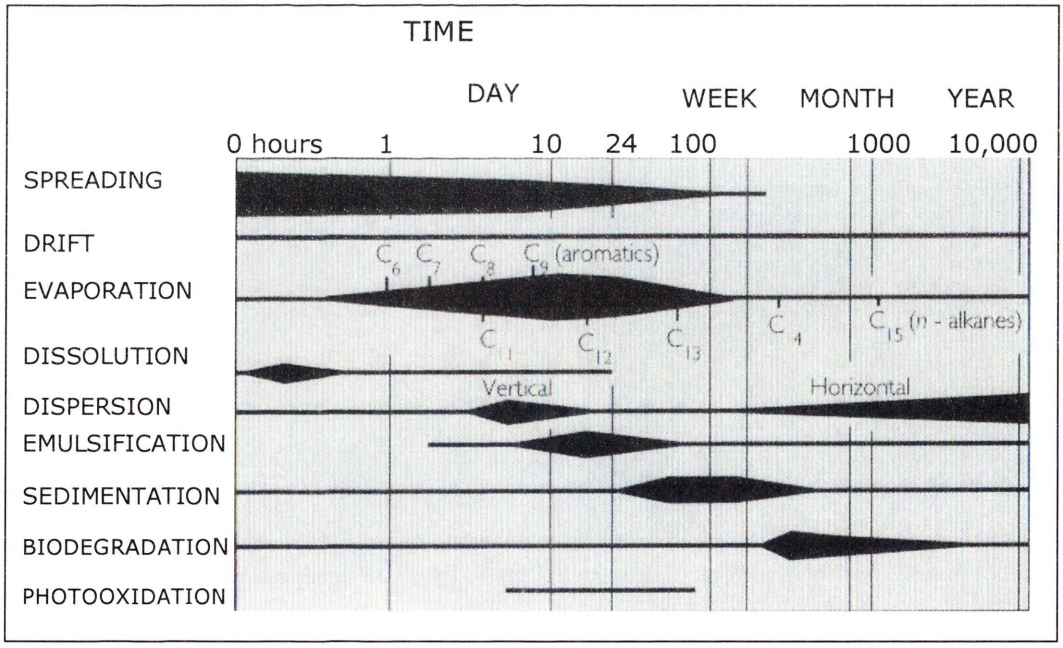

Figure 1.2: Diagram showing the timeline and relative importance of the weathering processes of spilled oil in the marine environment (adapted from Clark 2002).

In many oil spill events the appropriate mitigation technique becomes a question of "to disperse" or "not to disperse"?, with the decision having far–reaching ramifications. The choice of mitigation procedure will affect the habitats within the vicinity of the spill and also the associated flora and fauna within the subtidal seagrass meadow.

1.3.1 Leaving the oil to break down naturally

Leaving the oil to breakdown naturally can be an appropriate mitigation measure in certain circumstances, such as an oil spill occurring in deep off shore waters isolated from sensitive resources. In other instances there may be a clear threat to shoreline resources, but no method is available to stop the oil from coming to shore. There are no methods currently available to prevent an oil spill coming to shore in strong onshore wind conditions (AMSA 2004). In these instances the only option is to recover the oil after it has come to shore. An example of this was the *Pacific Adventurer* oil spill off Cape Moreton (Queensland), in 2009 (Table 1.1). The location, weather conditions at the time, and the oil type, meant there was considered no other appropriate mitigation procedure to deal with the oil, so the most appropriate action was deemed to leave the oil alone and allow it to wash to shore (Raaymakers 2009).

1.3.1.1 Effects on biota of non-dispersed oil

Although oil will undergo natural dispersion without the aid of chemical dispersants, in this thesis, unless stated otherwise, non-dispersed oil refers to oil that has not been chemically dispersed. The effects of non-dispersed oil on the biota are quite commonly of a physical, rather than chemical nature (Dodd 1974; Zieman *et al.* 1984; Jacobs 1988) and are usually manifested at the surface or in shallow water depths. Oil concentrations are highest closest to the surface and as a result those organisms living in intertidal or near surface water are generally most at risk (Jacobs 1988; Lee & Page 1997). Smothering or asphyxiation are a result of the physical effects of oils, but toxic effects

from the chemical uptake of the components within the oil can also occur. One of the main determining factors of organism response to non-dispersed oil is the amount of weathering the oil has undergone, which is dependent upon time, weather conditions, habitat of the organism and the potential for the organism to move away from the contamination (Lee & Page 1997; Singer *et al.* 2000; Gin *et al.* 2001).

Animals

Oil-contaminated mammals, birds, fish and reptiles are common following an oil spill (Clark 2002) and it is images of these that make media headlines and arouse public reaction. Birds are of particular concern, particular when their nesting colonies are located along the shoreline or where they risk ingestion of oil contaminated food sources (fish, invertebrates). Fairy Penguins and cormorants were contaminated with oil following the *Laura D'Amato* spill (1999) in Sydney Harbour (AMSA 2000). Following a heavy fuel oil spill in northern Tasmania, *Iron Baron* (1995), 1,894 little penguins, *Eudyptula minor*, required cleaning due to oil contamination. Seabirds and nesting birds are also at high risk of hypothermia when oil damages the thermal protection provided by their feathers (Clark 2002). Fish can be affected by oil spills through absorption of oil components or through asphyxiation due to smothering of their gills and juveniles are considered to be at most risk (Collier *et al.* 1993; Lee & Page 1997). Filter feeders such as oysters, polychaetes and amphipods uptake oil via feeding pathways, as well as being smothered (den Hartog and Jacobs 1980; Lee & Page 1997).

As the acute toxicity of non-dispersed oil is largely reliant upon the time following the spill until it reaches the organism, those species in the vicinity of the initial spill site are likely to be the most affected. Infauna associated with seagrass meadows can be severely impacted by oil, particular those in the intertidal, or those in the shallow subtidal with very heavy oiling (Jacobs 1988). The fauna associated with seagrass beds increases up to a few metres in water depth and then decreases (Van der Ben 1971; Jacobs & Huisman 1982), so the most complex region within the seagrass meadow is generally at less of a risk to long-term exposure of non-dispersed oil.

Changes in community structure are well documented following oil spills. Amphipod communities were devastated following the *Amoco Cadiz* (1978) spill but there were no, or minimal, affects to other fauna such as gastropods, isopods and echinoderms (den Hartog and Jacobs 1980). Opportunistic species such as polychaetes have been shown to thrive in regions following oil spills (eg. Smith & Simpson 1998; Nikitik & Robinson 2003). Seven years after the *Nella Dan* spill (1987) at Macquarie Island (sub-Antarctic), Smith and Simpson (1998) still found evidence of communities dominated by opportunistic worms in heavily oil impacted areas. Most sites at Macquarie island, however, returned to a community structure similar to non-oiled sites, suggesting that recovery had occurred (Smith & Simpson 1998), and is similar to other studies suggesting recovery to pre-spill conditions (Nikitik & Robinson 2003).

Plants

A variety of impacts have been documented following exposure to non-dispersed oil including retarding of seed germination and reductions in plant height, stem density, photosynthetic rate and biomass (Pezeshki & DeLaune 1993; Burridge & Shir 1995; Pezeshki *et al.* 2000; Melville *et al.* 2009). Exposure pathways of oils to plants include via direct contact, and, or uptake via the leaves or root system (Pezeshki *et al.* 2000; Dowty *et al.* 2001).

Plants coming into direct contact with oils are subjected to smothering which can reduce respiration and gas exchange between the water, and or air, and within the plant. Mangroves may be subjected to deleterious effects from contact with oils, as direct contact can smother the pneumatophores (Jackson *et al.* 1989; Tam *et al.* 2005). A high incidence of seedling mortality and defoliation was evident in mangroves in Port Curtis, following the *Global Peace* oil spill (2006) due to both oil smothering and oil residues in the sediments (Melville *et al.* 2009).

Phytoplankton within the water column and at the water surface are severely impacted by non-dispersed oils. Photosynthetic health clearly declined in a study investigating the

effects of PAH toxicity on the photosynthesis in microalgae (Huang *et al.* 1997). They detected differences using chlorophyll *a* fluorescence parameters and concluded the PAHs impacted on the photosystems, reducing the photosynthetic efficiency of the plants.

Many studies show a hormetic effect (enhancement of growth) at low concentrations of non-dispersed oil (Gordon & Prouse 1973; Karydis & Fogg 1980; Chan & Chiu 1985; Singh & Gaur 1990; Lin *et al.* 2002). Stimualtory effects on the growth rate and chlorophyll *a* concentrations of the green algae, *Chlorella salina*, were reported by Chan and Chiu (1985) from low concentrations of light diesel oil. The carbon component within the oil was thought to be assimilated into the plants and utilised for growth (Chan & Chiu 1985). Ruiz-Villarreal *et al.* (2006) found an increase in chlorophyll *a* concentrations in the water column immediately following the *Prestige* oil spill, however they did find it difficult to distinguish between this and natural variability. Whilst hormetic effects may not appear to be negative, there are some concerns regarding the possible long-term implications (Kefford *et al.* 2008).

Pezeshki *et al.* (2000) suggested any contact or uptake of oil will compromise the photosynthetic activities within a plant to some degree. However, oil contamination following the *Iron Baron* spill (1995) in Bass Strait, resulted in no significant effects to subtidal macroalgal species even though some oil was considered as having penetrated into the habitat (Edgar & Barrett 1995). This example is supportive of other studies suggesting minimal impacts to subtidal organisms from oil concentration.

1.3.2 Chemically dispersing the oil

Dispersant application is only considered when there is a serious risk posed to a resource (eg. a sensitive habitat) that may come into contact with the oil had the oil been left to disperse naturally; and when all environmental effects have been considered (Fingas 2001; AMSA 2007). Where prevailing winds or currents are likely to push an

oil slick onto a sensitive shoreline (eg. mangrove habitat, coral reef, nesting bird colony), the use of dispersants is considered (Fingas 2001). An example of effective dispersant application was following a spill of 72, 000 tonnes of light crude oil and 480 tonnes of heavy fuel oil, from the grounding of the *Sea Empress* spill (1996) in the United Kingdom (Lewis 2001). Dispersant application combined with natural processes reduced the amount of oil washing ashore to approximately 10 % of the initial spill volume. Although this spill devastated certain marine communities, the total environmental effect was considered far less than would have otherwise occurred had the oil not been dispersed and allowed to wash ashore (Lewis 2001).

Dispersants are a mixture of surfactants, solvents and stabilising agents (Fingas 2001). The application of a dispersant leads to the formation of small oil droplets in the water column effectively increasing the rate of breakdown of the slick (Lessard & Demarco 2000). A surfactant within the dispersant is amphiphilic, having a hydrophilic end (water compatible) and a lipophilic end (oil compatible) (Lessard & Demarco 2000). In essence, water molecules attach to the hydrophilic end, whilst oil molecules attach to the lipophilic end. This acts to decrease the interfacial tension between water and oil, effectively displacing the oil slick from the water surface into the water column (Lessard & Demarco 2000; Fingas 2001).

Apart from minimising the risk of contamination of shoreline resources, dispersed oil has further benefits that can reduce the impacts of a spill. Dispersing oil promotes microbial degradation of the oil by increasing the surface area; reduces the fire hazard of oil by increasing the rate of dispersion into the water column; does not allow oil to form into tar balls; and prevents oil adhering to bird feathers, pilings, boats or beach sand (Canevari 1979; Cintron *et al.* 1981; Lessard & Demarco 2000; Fingas 2001).

The effectiveness of dispersants is dependent on a variety of factors including the oil type, weather conditions and location of the oil spill (Lessard & Demarco 2000; Fingas 2001). Certain dispersants work better on different types of oils and in general, crude oil will commonly disperse better than bunker oil, due to its lower viscosity. For example,

some highly viscous, heavy fuel oils may not disperse well, or at all (Fingas 2001; Lewis & Lunel 2001).

1.3.2.1 Effects on biota of dispersed oil

Generation I dispersants were essentially industrial detergents and the poor usage of, and the risk posed by these became evident following the *Torrey Canyon* spill (1967) in the United Kingdom (Lewis 2001). Thirty thousand tonnes of Kuwait crude oil leaked within the first few days and approximately 75 tonnes of dispersant was sprayed onto the slick in attempts clean up the spill. The substantial ecological effects that followed were attributed to the disperant rather than any effects of the oil. Currently used Generation II dispersants, are much less toxic to aquatic biota than previous dispersants (Venosa & Holder 2007). Prior to approval for use in Australia, dispersants must first be tested on local fish and crustaceans from temperate and tropical waters. However, many studies still show negative effects of dispersants and dispersed oil on organisms (e.g. Ramachandran *et al.* 2004; Couillard *et al.* 2005; Shafir *et al.* 2007).

Animals

Many studies have shown that invertebrates, fish, particularly juveniles, and even corals, are impacted more when the oil is chemically dispersed (Ramachandran *et al.* 2004; Couillard *et al.* 2005; Shafir *et al.* 2007). The infauna of seagrass meadows can be severely impacted even when there is no impact to the seagrass itself (Zieman *et al.* 1984). Hatcher and Larkum (1982) suggested differences in respiration rates in dispersed oil-treated seagrass microcosms compared with non-dispersed oil treatments may have been due not from the seagrass, but from the impact to the epiphytic organisms. Wyers et al. (1986) showed similar findings with the coral *Diploria strigosa*. The coral itself was relatively tolerant to short term exposure (less than 24 hours) of physically and chemically dispersed oil, but fauna associated with the coral reef such as polychaetes and decapod crustaceans were negatively impacted (Wyers et al. 1986).

Plants

Thylakoid membranes within plants are made up of lipids, and dispersed oil is able to penetrate these photosynthetic components of plants. Similar to non-dispersed oil, hormetic effects are also common in plants at low concentrations of dispersed oil. Burridge and Shir (1995) showed that dispersed fuel oil was toxic to the marine macroalga *Phyllospora comosa*, however, dispersed crude oil under the same conditions showed a hormetic effect.

Mangroves are considered highly vulnerable to oil spills and chemical clean-up methods and as such a vast amount of research conducted on these habitats. Two prominent studies, The Gladstone Field trials (Burns *et al.* 1999) and The Tropics Study (Baca *et al.* 1996), showed different responses of the mangroves, but both led to similar mitigation recommendations. Burns *et al.* (1999) conducted a range of large-scale field trials, The Gladstone Field trials, assessing oil spill remediation in mangroves. They found dispersed oil led to significant negative impacts on the mangroves compared with non-dispersed oil. However, both non-dispersed and dispersed oil sites led to reductions in abundance of the associated fauna and the authors thereby concluded dispersing a spill prior to it reaching the mangrove system was the most viable option. The study by Baca *et al.* (1996) found dispersed oil led to some sub-lethal impacts to tropical mangrove systems, but this was far less than the mass mortality seen at the non-dispersed oil sites and so they recommended dispersing an oil slick prior to it reaching these vulnerable habitats.

1.3.3 Net Environmental Benefit Analysis

The Australian Maritime Safety Authority is the Australian agency responsible for determining the appropriate mitigation procedures to be applied following an oil spill. Current recommendations by the National Plan Environmental Working Group are to not use dispersants in less than 5 - 10 m of water because of the increased risk this poses

to subtidal organisms. In waters less than 5 m deep, the Oil Spill Coordinator (OSC), however, may choose to disperse an oil spill when the risk posed to another resource would be far greater than had the oil not been dispersed. To determine this, a Net Environmental Benefit Analysis (NEBA) is used.

The Net Environmental Benefit Analysis (NEBA) is an assessment of the overall impacts to the resources within the oil spill environment (Lunel & Baker 1999; Baca *et al.* 2005). It is conducted to determine the least net environmental affect arising from mitigation strategies or options being considered. Whether to disperse or not an oil spill, then usually becomes a "trade- off" between shoreline and subtidal resources (Lessard & DeMarco 2000; Fingas 2001). The impact on subtidal organisms, such as subtidal seagrass meadows, may have been minimal if any, had the oil not been dispersed, as the oil slick simply may have passed over the top, whereas intertidal and shoreline habitats may benefit more if the oil is dispersed by reducing the amount of oil reaching these habitats.

Subtidal seagrass are considered at less of a threat to oil contamination than, for example mangrove habitats, because of the limited ability of oil reaching the depth of their habitat. However, subtidal seagrass are still potentially vulnerable to the impacts of oil pollution (Zieman *et al.* 1984; AMSA 2008) and oil spill events within the vicinity of subtidal seagrass meadows are common. The determination of a NEBA when a spill has occurred within an area that has both subtidal seagrass meadows and sensitive shoreline habitats (eg. mangrove habitats, coral reefs) becomes complicated as even though there appears a greater risk to the shoreline habitat, the seagrass may suffer damage with the choice of mitigation procedure. The lack of knowledge of the effects of dispersants to seagrass (AMSA 2008) is likely to compromise the mitigation decision making process following an oil spill in these complex habitats.

1.4 Oil Spill Research and Subtidal Seagrass

Research has been conducted on the effects of petrochemical contamination to seagrasses in laboratory and *in situ* experiments, whilst other studies have been conducted following real oil-spill events. Of the studies conducted, however, there is some disparity as to whether non-dispersed or dispersed oil poses the greater threat to seagrass, and the findings of laboratories studies may not represent the findings following real oil spill events.

1.4.1 Effects of non- dispersed oil on seagrass

The body of evidence to suggest seagrasses are impacted by oil is, comes from when there has been a direct contact with the above ground biomass of the plant, the blades (Jacobs 1988). Even so, unless the oil is retained within the seagrass meadow for a sustained time most studies report no long- term impacts (Zieman *et al.*. 1984; Jacobs 1988). As stated previously, the impact from oil alone is quite commonly related to the physical problem of the oil smothering, rather than the chemical toxicity.

Oil coming into direct contact with seagrass blades has been shown to negatively affect the plant (Jacobs 1988; Jackson *et al.* 1989). Oil by itself affects seagrasses through the adsorption of the water accommodated fraction (WAF) which leads to a reduction in tolerance to other stress factors (Zieman *et al.* 1984). Smothering and fouling are some of the effects that have been documented from oil contamination (Blumer 1971; Cintron *et al.*. 1981). Seagrass blades become bleached, blackened, yellowed and detached from the plant following direct oil contamination (Chan 1973; den Hartog & Jacobs 1980; Dean *et al.* 1998; Jackson *et al.* 1989). Other affects from direct contact include a decrease in the density of shoots and flowering shoots (Chan 1973; den Hartog & Jacobs 1980; Dean *et al.*. 1998). Zieman *et al.* (1984) suggested that in most cases the system has the ability to recover however; damage to the rhizome-sediment structure may result in irreversible damage as sediment stability becomes compromised.

Simulated oil spill experiments provide somewhat conflicting evidence, but have generally shown minimal impact to the seagrass investigated. Hatcher and Larkum (1982) found minimal stress placed on *Posidonia australis* due to exposure to non-dispersed Bass Strait crude oil; Durako *et al.* (1993) found no significant impacts on three species of seagrass exposed to Kuwait crude oil; Macinnis-Ng and Ralph (2003a) showed a severe short-term (six hours) decline in the effective quantum yield of *Z. capricorni* following exposure to the water soluble fraction (WSF) of crude oil; Ralph and Burchett (1998b) found the WAF of Bass Strait crude oil caused only minor impacts to the seagrass *Halophila ovalis*; whilst Thorhaug *et al.* (1986) detected impacts on tropical seagrass species due to non-dispersed oil, but this was less than that evident from dispersed oil treatments. There were no significant affects on the seagrass exposed to heavy fuel oil spilt in Gladstone Harbour, Queensland, in 2006 (Taylor *et al.* 2006). Aston (2006) reported small patches of dead seagrass following the heavy fuel oil spill in Port Curtis, Queensland, in 2006, however Taylor *et al.* (2006) found no significant differences between oiled and control sites at the same location. Several studies investigating the long-term affects of major oil spill incidents have also suggested minimal impact. Baca *et al.* (1996) found no significant impacts on tropical seagrass during a ten-year monitoring study of a simulated crude oil spill (Baca *et al.* 1996). Studies investigating the affects on seagrasses from the Gulf War spill in Saudi Arabia (Kenworthy *et al.* 1993) and the Exxon Valdez spill in Alaska (Dean *et al.* 1998) generally found some differences several months following the incidents, but no significant differences one year afterwards.

Subtidal seagrass can still be subjected to, and impacted by, direct contact with oil that has not been chemically dispersed. Subtidal beds of *Thalassia* and associated fauna were decimated following a crude oil spill in Puerto Rico in 1973 (Naduau & Berquist 1977). Strong weather conditions caused the downwards entrainment of oil into the subtidal seagrass beds. The crude oil in this spill was considered to be of low toxicity, yet the impacts to the seagrass were so severe that even the rhizome layer was affected. The seagrass had shown some evidence of recovery three years following the spill (Naduau & Berquist 1977), but such an impact is of concern as large, slow growing

species such as *Thalassia,* are considered more resilient than smaller faster growing species (Cheshire *et al.* 2002).

Most studies assessing the effects of oil and dispersed oil on seagrass have focused on crude oil (eg. Hatcher & Larkum 1984; Thorhaug *et al.* 1986; Baca *et al.* 1996; Ralph & Burchett 1998b; Macinnis & Ralph 2003a). No manipulative studies addressing the effects of fuel oil on seagrass could be found by the author. Some actual spill events of fuel oil could be found with reference to seagrass impacts, but as in the case of the *Amoco Cadiz* spill of 1978 (Jacobs 1980) the spilt oil was a combination of both crude and fuel oil, and furthermore there was only little reference to the actual seagrass itself, with an emphasis of the impact on the infauna. Tam *et al.* (2005) noted similar findings with respect to mangrove contamination in that contamination by fuel oil received little attention compared with crude oils. Considering the recent spate of fuel oil spill incidents in Australian waters (eg. *Pacific Adventurer* (2009), *Global Peace* (2006)), an understanding of the affects of these oils to subtidal seagrass is required if mitigation procedures are to be guided.

1.4.2 Effects of dispersed oil on seagrass

Dispersed oil is considered to increase the risk to subtidal seagrass but similar to non-dispersed oil, studies have shown a variety of findings. Seagrasses have been shown to absorb more aliphatic and aromatic oil fractions when the oil is dispersed, therefore increasing the toxicity (den Hartog 1984). Dispersants are thought to affect the waxy cuticle of the seagrass blade and in so doing increase the penetrability of the dispersed oil to the photosynthetic organs, particularly the thylakoid membrane (Howard *et al.* 1989; Wolfe *et al.* 1998). Dispersed oil leads to greater microbial breakdown which can lead to a heavier (greater) oxygen demand by the microbes (Fingas 2001; NRC 2005). A reduction in the oxygen available to the seagrass community may impact on the seagrass system because as seagrasses have a high respiratory demand to support their large non-photosynthetic underground biomass (Zieman *et al.* 1984).

Macinnis and Ralph (2003a) found only minimal impact to the photosynthetic efficiency of *Z. capricorni* when exposed to dispersed oil *in situ*. In the same study, the dispersant VDC alone similarly led to minor impacts *in situ*, however, in laboratory experiments, the dispersant caused a delayed impact following the replenishment with 'fresh' seawater with no signs of recovery after four days. Hatcher and Larkum (1984) found that dispersed oil reduced the respiration rate of *Posidonia australis,* but only marginally. They further suggested that a reduction in epiphytic organisms may have reduced the overall respiration amount within the microcosm and furthermore, the reduction of water clarity due to the dispersed oil may have played a role in reducing the respiration rate of the plants (Hatcher & Larkum 1984). Thorhaug *et al.* (1986) found dispersed oil had a greater affect on tropical seagrass species than non-dispersed oil. Thorhaug *et al.* (1986) showed that the species investigated differed in their sensitivities to the petrochemicals, whilst Thorhaug and Marcus (1985) found that toxicity was partly dependent on the type of dispersant used. The long-term study by Baca *et al.* (1996) showed no adverse effects to tropical seagrass by dispersed oil. Simarlarly, following the *World Encouragement* spill of 1979 in Botany Bay, and the subsequent dispersant application, no obvious effects to the seagrass were found (Larkum & West, unpublished report 1981 - In: Larkum & West 1990).

Although most research implies minimal impact to seagrass from oil and dispersed oil (eg. Baca *et al.* 1996; Ralph & Burchett 1998b), the fact that some research has shown seagrass to be detrimentally impacted (eg. Nadeau & Berquist 1977; Thorhaug *et al.* 1986) suggests that under certain conditions seagrass can be adversely affected by petrochemicals. Why different studies have found different levels of response from seagrass may be due to a number of different factors, including, different methodologies being used, variation in species being investigated, oil and dispersant chemical variation, and differences in the loading volumes exposed to the seagrass. Perhaps this disparity is more to do with the experimental design of each study, rather than the disparity in the seagrass response.

1.4.3 Disparity in research findings

Singer *et al.* (2000, p. 1008) stated with regards to petrochemical exposure analyses that "comparisons among species, life stages or toxicants are difficult, if not impossible, when there are significant differences in the methodologies used to produce data". The previously described research has many differences. Seagrass health in the studies detailed above were derived by a variety of methods including Pulse Amplitude Modulated (PAM) fluorometry and photosynthetic pigment analyses (Ralph & Burchett 1998b; Macinnis & Ralph 2003a); oxygen production and respiration techniques (Hatcher & Larkum 1984; Durako *et al.* 1993); growth techniques (Thorhaug *et al.* 1986); and density measurements (Kenworthy *et al.* 1993; Baca *et al.* 1996; Dean *et al.* 1998). Thermal conditions of the simulated, or actual, oil spills were also often different. Higher temperatures enhance the solubility of hydrocarbons and the rate of their degradation through microbial activity (Burridge & Shir 1988) whilst many seagrass species show different growth patterns in different seasons (Kirkman *et al.* 1982; Larkum *et al.* 1984). Furthermore, tropical species tend to have faster growth rates and respond differently to change than temperate species (Waycott *et al.* 2005). Morphological variety in seagrass is vast and species resilience to petrochemical impacts is likely to reflect this. Thorhaug *et al.* (1986) showed clear differences in the response of different species of tropical seagrass to petrochemicals but to date, other research has largely been conducted on single species (eg. Baca & Getter 1984; Hatcher & Larkum 1984; Ralph & Burchett 1998b; Macinnis & Ralph 2003a). Different species of seagrass clearly respond differently to stressors, including those imposed by petrochemical pollution. Most studies, however, do not incorporate this into their research design due likely due to the increased logistical effort of conducting multi-species analyses, specifically with aquatic macrophytes (Kuster & Altenburger 2007).

1.4.4 Field compared with laboratory experiments

Field assessments are commonly logistically intensive in time, cost and effort. Küster and Altenburger (2007) stated the logistical difficulties of toxicological tests involving

aquatic macrophytes because of the slow growth of species and the high amounts of toxicants required to produce a significant response. However, there is a disparity between results obtained from real spill events, *in situ* experiments and laboratory experiments particularly with the effects to subtidal seagrass (eg. Macinnis & Ralph 2003a). Laboratory experiments have been shown to overestimate the effects of real spills and *in situ* experiments, but they are the least logistically intensive to conduct (Clark & Noles 1994; Macinnis and Ralph 2003a). Many environmental variables are difficult to replicate in the laboratory such as light attenuation into the seagrass canopy but field experiments often lack environmental control producing inconclusive results (Hemminga & Duart 2000; Clark & Noles 1994). Laboratory experiments require field validation, but this is commonly lacking in most studies.

1.4.5 Application of a rapid laboratory testing protocol

One underlying theme in an oil spill event is that there are a multitude of factors that come into play once an oil spill has occurred (Loya & Rinkevich 1980) which determine how a community responds. As stated throughout this chapter, oil type, oil concentration and weather conditions to name but a few, are some of the factors that will determine the risk to the organism community in question. Asseessments of pollutant impacts to organisms are commonly conducted using 96 hour static exposure tests (e.g. as recommended by USEPA). Considering the many different oils and dispersants currently available coupled with the variety of seagrass species and the multitude of environmental variables, many combinations of these will never be studied based on the 96 hour USEPA static renewal tests.

Furthermore, the Window of Opportunity is the period between the oil spill occurring and the time when it is no longer viable to disperse, ranging from several hours post spill to greater than one day (NRC 2005). In Australian waters, the Window of Opportunity is further limited by the expanse of the Australian coastline and the increased time required for gear and dispersant deployment in isolated areas

(Lipscombe 2000). This limited time period, greatly increases the requirement of having sound information readily available, on the effects of oil and dispersed oil on seagrasses within the vicinity of the spill.

Reflecting on the two factors highlighted from the above points, 1) the many factors determining the effect of spilled oil to seagrass, and 2) the short time period post spill in which dispersant application is feasible, suggests a more rapid approach to determining seagrass response from petrochemicals would be beneficial. A rapid testing protocol would allow for more species to be assessed and more oil and dispersed oil combinations to be conducted. Furthermore, the ability to conduct these tests under conditions such as different water temperatures would greatly increase our knowledge of the potential effects to the seagrass. If the exposure time were short enough, then more experiments could be conducted prior to an oil spill occurring, but experiments could also be conducted immediately following an oil spill. Rapid tests conducted following an oil spill may assist with determining the appropriate mitigation procedure for that particular seagrass meadow. The more information that can be determined regarding the impacts of oil and dispersed oil to the seagrass, the greater the confidence for oil spill mitigation managers in determining the best mitigation procedure.

1.5 Significance of this study

An increase in research has been conducted on seagrass and petrochemical impacts in the past ten years. However, the question still remains whether oil or dispersed oil poses a threat, or one poses a greater threat than the other, to subtidal seagrass. Oil spill clean-up managers are still limited in their use of dispersants around subtidal habitats due to this uncertainty. Field testing methods are logistically intensive but provide the most realistic results in terms of impacts. Laboratory methods are thought to overestimate the effects of petrochemicals (Macinnis & Ralph 2003), but in some cases they do allow for a wider variety of combinations to be investigated, and often require less economic and logistical effort than field experiments. The study reported in this thesis aimed to

incorporate methods used in previous studies to form a more comprehensive picture of the relative effects of oil and dispersed oil. This study addresses seagrass response to oil and dispersed oil, by assessing whole plants *in situ* and, whole plants and sections of leafblades under laboratory conditions.

This is the first study to comprehensively assess the response of *Zostera capricorni* and *Halophila ovalis* to a crude and heavy fuel oil oil, *in situ* and under controlled laboratory conditions. This is also the first study in developing a rapid testing protocol for detecting petrochemical impacts on subtidal seagrass.

1.6 Aims

The major aims of this project were to determine:

1) the extent to which subtidal seagrass is affected by non-dispersed and dispersed crude and heavy fuel oil and at what concentrations;

2) the relative effects of different temperate seagrass species to oil and dispersed oil;

3) whether *in situ* experiments can be replicated in laboratory experiments; and

4) whether leafblade sections can be used as a rapid assessment of seagrass stress from petrochemicals.

2 General Methods

This chapter describes the methodologies which are common throughout the thesis, or have relevance for understanding of, the subsequent chapters.

2.1 Field Sites

Bonna Point, Botany Bay, New South Wales

The northern study site, Bonna Point (151° 11' E, 34° 00' S) is located along the southern end of the Kurnell foreshore, Botany Bay, Sydney (Figure 2.1). Botany Bay is a shallow marine bay approximately 49 km^2 in area (4600 ha Larkum & West 1990), located at the entrance of the Georges River (Bell & Westoby 1986; West *et al.* 1990). Towra Point Aquatic Reserve is located on the southern edge of the bay, adjacent to the study site (Figure 2.2). The Reserve is classified as a Ramsar Wetland of International Significance due to the abundance of important habitats supporting high biodiversity (Department of the Environment, Heritage and Arts 2010). The reserve contains about 50 % of the remaining mangrove communities and about 90 % of the remaining saltmarsh communities within the Sydney region. The seagrass beds, in conjunction with its mangrove and saltmarsh communities, provide critical shelter and food for juvenile fish and crustaceans (Department of the Environment, Heritage and Arts 2009). Threatened bird species, such as the Little Tern (*Sterna albifrons*) and Pied Oystercatcher (*Haematopus longirostrus*) are known to breed within the area and threatened plants including Magenta brush cherry (*Syzygium paniculatum*) inhabit the reserve (SOE 1996; Department of the Environment, Heritage and Arts 2010).

Three species of seagrass are present along the southern edge of Botany Bay, *Posidonia australis*, *Zostera capricorni* and *Halophila ovalis*. *Posidonia australis* is found within

the deeper water, with the shallower sections of the bay largely colonised by *Z. capricorni* (Larkum & West 1990; West *et al.* 1990; Creese *et al.* 2009). *Halophila ovalis* is commonly found growing intermittently within the *Z. capricorni* and *P. australis* meadows, but there are some distinct monospecific meadows along the northeastern edge of the bay (West 1990; Creese *et al.* 2009). Seagrass decline has occurred in Botany Bay with Larkum and West (1990) reporting a 58 % (257 ha) loss of *Posidonia* beds between 1942 and 1984 due to both natural (e.g. storm events) and anthropogenic affects. Larkum and West (1990) noted that *Z. capricorni* had colonised areas previously inhabited by *Posidonia*.

Botany Bay is the location of many large-scale industrial activities including a petroleum refinery and the Port of Botany supports major shipping activity with a container terminal. These activities have increased the risk of petroleum contamination to seagrass and major oil spills have occurred in previous years (see Table 1.1). Other industrial activities include the Kingsford Smith airport and the Sydney Desalination Plant. Extensive dredging during the construction of these and other activites has likely increased disturbance to the seagrass in such ways as altering flow regimes and increasing the turbidity in the water column (Larkum & West 1990).

Corio Bay, Port Phillip Bay, Victoria

The southern study site, Corio Bay, is situated along the western edge of Port Phillip Bay. Port Phillip Bay has an area of about 2000 km^2 with a maximum depth of 25 m (Smith & Maher 1984; Zann 1996). Port Phillip Bay supports major shipping and industrial activity including an oil refinery. Meadows of *Zostera muelleri* are present close to shore and extend into the deeper waters of Corio Bay. The seagrass meadows within the bay support important recreational and commercial fish and invertebrate species such as King George Whiting (Officer & Parry 1997). Annual surveys of juvenile King George Whiting are conducted in Corio Bay to assist with the determination of commercial fishing catch limits within Port Phillip Bay.

Several major oil spills have occurred within Port Phillip Bay. Smith and Maher (1984) determined, however, hydrocarbon contamination within the sediments of Corio Bay was largely derived through shipping activity (Smith & Maher 1984). High levels of nutrients occur within the bay (Zann 1996) with discharges of stormwater (Ghafouri & Swain 2005) and large amounts of decomposing seagrass present on the shoreline.

2.2 Seagrass Methods

2.2.1 Seagrass collection & culturing

Healthy samples of *Zostera capricorni* and *Halophila ovalis* were collected from Bonna Point, Botany Bay, for laboratory culturing and experiments. *In situ* experiments and the collection of seagrass were performed under a New South Wales Department of Primary Industry Scientific Research Permit (Permit numbers: P06-09/0010). Seagrass was collected with rhizomes and sediment attached to reduce disturbance to the plant. This was performed by hand and all samples were placed into plastic bags. The bags were half filled with seawater and were immediately transported to the UTS laboratory for transplantation.

Seagrass was transplanted into plastic trays (40 mm H x 80 mm L x 50 mm W) containing approximately 3 cm of the sediment from the collection site and a 1 cm top layer of sand which was used to prevent blooms of microbes occurring at the sediment - seawater interface. Seagrass was maintained in a 50 L flow-through aquarium under 150 µmol photon $m^{-2}s^{-1}$, with a 16: 8 h dark: light photoperiod. Two sump pumps (Jun Aquarium 220v) cycled the water through the aquaria and the filtration system. The filtration system consisted of foam sheeting and bioballs (Polytech, Australia) that assisted in removing particulates and excess nutrients from within the aquaria. Several small air stones assisted in oxygenation of the water within the tank. pH was maintained at 7 ± 0.5, monitored using a portable pH probe. Evaporation from the aquarium was counteracted by a deionised water–filled header tank that replenished the water in the sump via a gravity-fed system. Water changes (saltwater) of approximately 20 L were

conducted between one and two times per week. Saltwater was supplied via the UTS laboratory storage supply sourced from Rose Bay (Sydney Harbour) and UV filtered. Seagrass blades were 'wiped' of epiphytic algae by hand several times per week and were acclimated in the aquaria for ten days prior to use in the laboratory experiments.

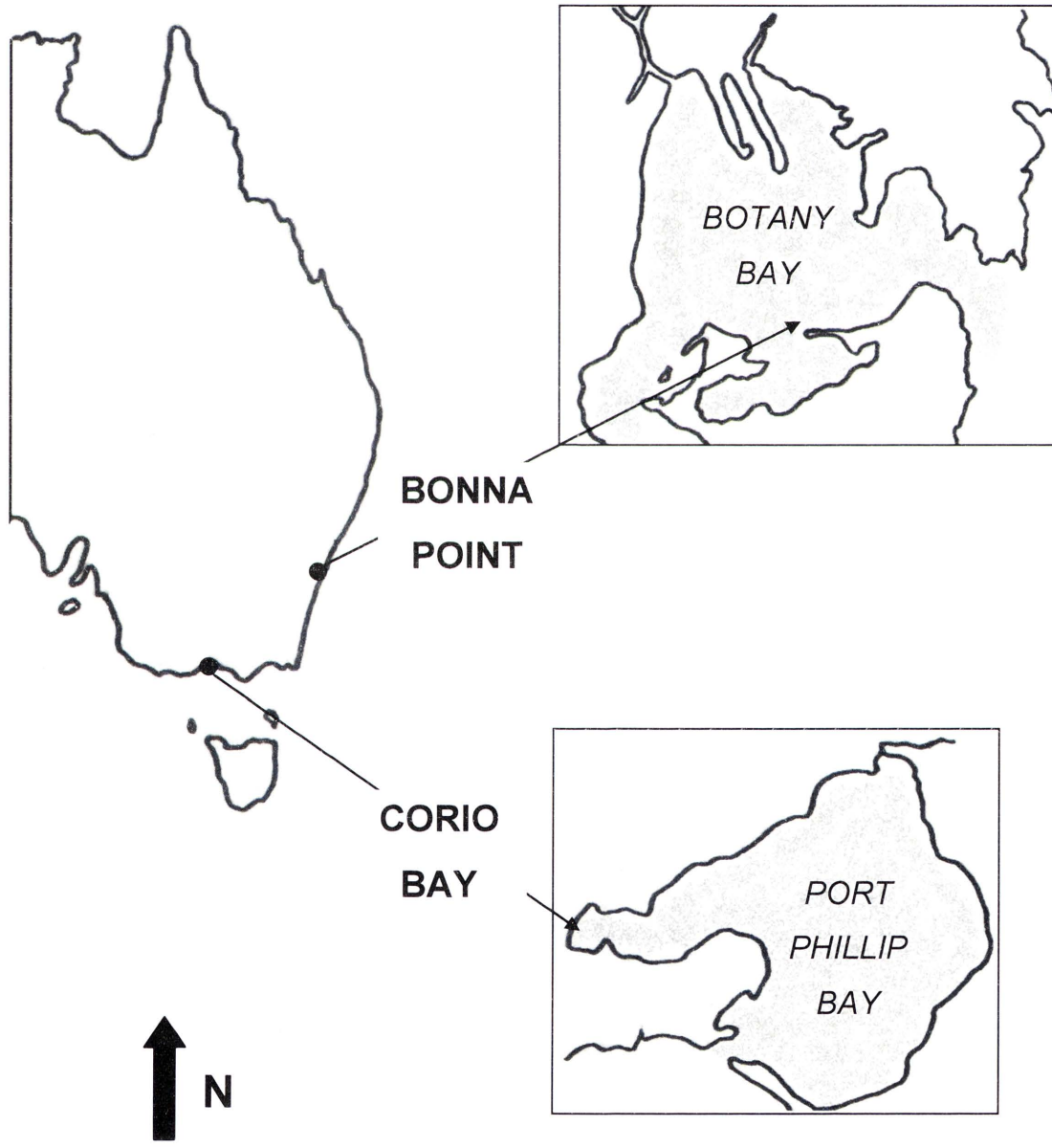

Figure 2.1: Location of Bonna Point in Botany Bay, New South Wales; and Corio Bay in Port Phillip Bay, Victoria; Australia.

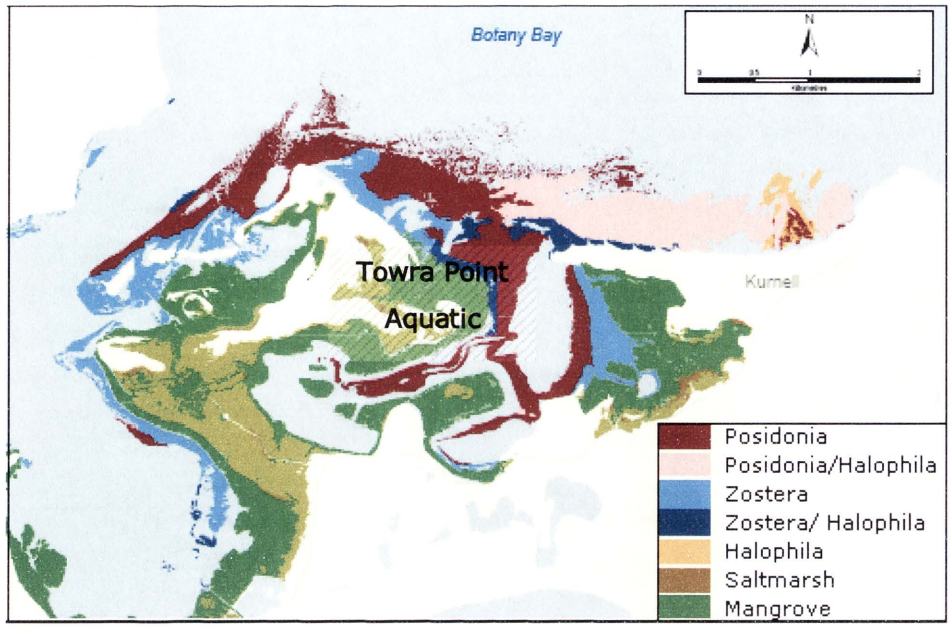

Figure 2.2: Map showing distribution and composition of seagrass, saltmarsh and mangrove habitats along the southern side of Botany Bay, Sydney and the general location of the Towra Point Aquatic Reserve of RAMSAR significance (Adapted from Creese *et al.* 2009).

2.2.2 Description of seagrass species

The two 'species' formerly termed *Zostera muelleri* and *Zostera capricorni* have recently been revised and are now considered subspecies of *Zostera muelleri* (Jacobs *et al.* 2006). The species name, *Zostera muelleri*, was given priority for taxonomic purposes reflecting the earliest name for the group (Jacobs *et al* 2006). There are considered some minor, but weak, morphological differences (leaf tip and seed surface morphology) between the two subspecies but these are considered a function of the location of the habitat (eg. depth limit, temperature) rather than a taxonomic difference (Les *et al.* 2002; Jacobs *et al.* 2006). Throughout this thesis, the two subspecies, *Zostera muelleri* subsp. *capricorni* (*Asch.)* S.W.L. Jacobs and *Zostera muelleri* subsp. *muelleri* Irmisch ex Ascherson, are referred to as *Zostera capricorni* and *Zostera muelleri*, repectively. This was employed for simplicity purposes and to incorporate research conducted prior to the revised classification system. Much of the literature referred to in

this thesis was published prior to the revised classification system, and refers to *Z. capricorni* and *Z. muelleri* as two distinct species.

Zostera muelleri subsp. *capricorni* (Ascherson) S.W.L. Jacobs
(Family Zosteraceae, Genus Zostera)

Zostera capricorni (Figure 2.3a), commonly called "eelgrass" or "garweed", is one of the most common species of seagrass in eastern Australia (Conacher *et al.* 1994; Brenchley *et al.* 1998). The subspecies is considered tropical or warm temperate, with its distribution ranging from tropical Queensland to as far south as Mallacoota, Victoria, and it is also found along the coasts of Lord Howe Island and New Zealand (Womersley 1984). *Zostera capricorni* forms extensive beds and has a depth range to about 7 m. The leaves are long, slender and strap-like, 70 – 500 mm long and 2.5 to 5 mm wide (Womersley 1984). The blades of the seagrass can be covered with much epiphytic algal growth. Flowering occurs between September and February, but has been recorded throughout the year by Harris *et al.* (1980) in Illawarra Lake, New South Wales. At Bonna Point, while *Z. capricorni* has shown a decline in coverage, it has been recorded that the species has recolonised over once inhabited by *Posidonia australis* (Larkum & West 1990). Juveniles of economically important fish species occurring within the *Z. capricorni* meadows in Botany Bay include bream, luderick, flathead and leatherjacket (Middleton *et al.* 1984). Seasonal differences in fish abundances were apparent and high abundances during late spring and early summer were attributed by Middleton *et al.* (1984) to the timing of regrowth of *Z. capricorni* following winter dieback. Similarly, Larkum *et al.* (1984) reported a winter die-back of *Z. capricorni* in Botany Bay with a four-fold reduction in shoot biomass compared with summer.

Zostera muelleri subsp. *muelleri* Irmisch ex Ascherson
(Family Zosteraceae, Genus Zostera)

Zostera Muelleri (Figure 2.3b) is distributed from the Yorke Peninsula, South Australia, along the southern coastline of Australia and Tasmania, to as far north as Sussex Inlet, New South Wales, and in New Zealand waters. The blade is linear, like *Z. capricorni*,

and is commonly 40 to 100 mm long, but can grow up to 600 mm long, and is 1-3 mm broad. Seasonal growth patterns of *Z. muelleri* are similar to that of other Australian Zosteraceae species in having a higher leaf growth rate during summer than winter (Kerr & Strother 1990) and flowering generally occurs between October to March (Womersley 1984).

Halophila ovalis
(Family Hydrocharitaceae, Genus Halophila)

Halophila ovalis (Figure 2.3c) is widey distributed in tropical and warm temperate waters of the Indo-Pacific region. It is found commonly along the western Australian coast and down the eastern Australian coast to the Victorian border (Womersley 1984; Kirkman 1997). The common name, "paddle grass", is derived from the oblong to obovate leaves, 10 to 40 mm long and 5 to 20 mm wide (Womersley 1984; Green & Short 2003). Flowering and fruiting occurs between August and April. The species forms extensive beds and is known to exhibit much morphological variation (Green & Short 2003).

2.2.3 Chlorophyll *a* fluorescence

To assess photosynthetic impacts to the seagrass from the petrochemicals, the effective quantum yield ($\Delta F/F_m'$) of the seagrass was determined using Pulse Amplitude Modulated (PAM) fluorometry. The $\Delta F/F_m'$, a measure of the health of photosystem II in light, provides information regarding active and inactive photosynthetic apparati. The technique thereby, provides information on the photosynthetic efficiency of the plant (Schreiber *et al.* 1994).

The effective quantum yield ($\Delta F/F_m'$) of Photosystem II (PSII) = $((F_m' - F_t)/ F'_m)$

Where:

- ΔF = Change in fluorescence
- F_t = Steady state fluorescence yield
- F_m' = Light - adapted maximum fluorescence

Figure 2.3: Images of the seagrass species investigated in this study; a) *Zostera capricorni*, b) *Zostera muelleri*, and c) *Halophila ovalis*.

The $\Delta F/F_m'$ is calculated by determining the ratio of variable fluorescence in a light-adapted state (ΔF) to maximum fluorescence in a light-adapted state (F'_m) (Schreiber & Bilger 1993; Govindjee 1995). Variable fluorescence (ΔF) is the difference between steady state fluorescence (F_t) and the maximum fluorescence in a light-adapted state (F'_m) (Maxwell & Johnson 2000). The maximum fluorescence (F'_m) is induced by a short saturating pulse of light, oxidising the reaction centres (Schreiber & Bilger 1993).

A Diving PAM and Mini PAM (Walz, Germany) were used for *in situ* and laboratory measurements, respectively. The Diving PAM has a submersible housing allowing underwater operation but is somewhat cumbersome in laboratory experiments, and the Mini PAM was used instead. The Diving and Mini-PAM operational settings were as follows: measuring light intensity: 10; saturating pulse intensity: 8; saturating pulse width: 8; gain: 8. PAM measurements were taken from the seagrass blade using a 2 mm optic fibre fixed in place for the duration of the exposure period (further descriptions are provided in the relevant chapters).

PAM measurements were taken from the second leaf blade approximately 5 cm above the apical meristem in *Z. capricorni* and *Z. muelleri* and in the middle of the adaxial surface in *H. ovalis* (Macinnis & Ralph 2003b; Ralph *et al.* 2005)

2.3 Oil Description, Preparation and Analysis

2.3.1 Description of oils and dispersants

The oils and dispersants used in this study were supplied by BP Australia and Caltex Australia, and were sourced via the Australian Maritime Safety Authority and the National Plan Environmental Working Group.

Tapis crude oil

Tapis crude oil was originally sourced from the Tapis region in Malaysia, and is commonly transported in Australian waters (Department for Planning and Infrastructure

2007). The oil is a light to medium crude oil with a low sulphur content (0.02 %) compared with other crude oils (Environment Canada 2001). The API (American Petroleum Industry) gravity is a measure of the relative density of oil compared to water and is measured in degrees. Tapis crude oil has an API gravity of about 45 degrees and viscosity of 2.7 at 30 °C (Department for Planning and Infrastructure 2007), indicative of a low density and the ability of the oil to float on the water surface.

IFO-380

IFO-380 (Intermediate Fuel Oil) is a common residual fuel oil used throughout the world, and is the fuel oil used to power the majority of large merchant ships (Lunel & Lewis 2001). Although composition varies, IFO-380 is highly viscous, 380 cSt at 50°C and has an API gravity of about 19 degrees (Exxon Mobil 2001; Lunel & Lewis 2001; Department for Planning and Infrastructure 2007). Due to the high viscosity, evaporation and dispersion of IFO-380 in the marine environment occurs at a slower rate than crude oils and the oil does not generally decompose at ambient temperatures (Ansell *et al.* 2001; Lunel & Lewis 2001).

Dispersants

All dispersants used within this study are recommended for use in Australian waters and are classified as Type II/ III (AMSA 2006). Corexit ® 9527 (ExxonMobil, USA) and Ardrox 6120 were used to disperse Tapis crude oil, whereas Slickgone LTSW and Corexit® 9500 (ExxonMobil, USA) were used to disperse the 380cSt fuel oil. In the figures and tables within this thesis the Corexit dispersants are referred to as C9527 and C9500, whereas Ardrox 6120 and Slickgone LTSW are referred throughout as simply Ardrox and Slickgone.

2.3.2 Preparation of the water accommodated fraction (WAF)

As the water accommodated fraction of oil is the component which has the potential to affect the subtidal organisms, the methods associated with the preparation of the water

accommodated fraction need to be followed carefully (Singer *et al.* 2000). Singer *et al.* (2000) provided guidelines for optimal and replicable weathering procedures based on extensive research. They found that difficulties arose when comparing species response with petrochemicals chiefly because of differences in the preparation of the water accommodated fraction (WAF). They recommended weathering via stirring for no greater than 24 hours in dark, temperature-controlled conditions (Singer *et al.* 2000). Others such as Thorhaug *et al.* (1986) and Clarke (2001) used and recommend pan weathering, whereby the oil is placed in shallow vessels (<10 cm) and left in the sun for evaporation. This is considered more representative of natural weathering with evaporation of the lower weight components from a floating slick on the surface. However, logistical difficulties can arise with this method. Large volumes of WAF would require a large surface area exposed to natural sunlight for pan weathering and space may be limiting at some laboratories. Also, different rates of oil weathering are likely to occur with this method due to naturally changing environmental conditions that cannot be experimentally controlled.

Five litres of filtered (0.45-μm) seawater brought to summer average seawater temperature (Sydney), 22° C (Bureau Of Meteorology 2009), was held in 5-L Erlenmeyer flasks and placed on magnetic stirrers (150 rpm). A vortex was created prior to the addition of oil to decrease the amount of oil forced onto the inside flask wall (Singer *et al.* 2000). Tapis crude oil and 380cSt fuel oil (Caltex Australia Petroleum) were added close to the water surface using a glass pipette. To produce a 1 % w/v solution, 50 g of oil was added to the 5 litres of seawater. During weathering, the room was darkened to reduce oil degradation and alteration by artificial lighting (Singer *et al.* 2000).

The water was stirred for 24 hours in the flasks and allowed to settle for one hour. For the dispersed oil experiments, 5 g of dispersant (Caltex Petroleum Australia) was added and the water stirred for a further 10 minutes prior to settling for one hour. The flask lids were removed, plastic tubing was inserted below the oil - water surface and the WSF was siphoned into amber glass bottles using a vacuum pump. The WAF was held at 4° C in darkness and used within two days (Singer *et al.* 2000).

2.3.3 Chemical analysis of the water accommodated fraction

Oils have been described as one of the most complex and variable mixtures to study toxicologically posing a major challenge to analytical chemists (UNEP 1992; API 1999; Singer *et al.* 2000). No single method of analysis will detail the entire components within an oil (UNEP 1992). The most descriptive and accurate methods include gas chromatographic (GC) and high performance liquid chromatography (HPLC). However, due to the time and expense of these two methods other more rapid and less logistically intensive methods have been devised (Lambert *et al.* 2003; Kim *et al.* 2010). Considering an oil spill needs to be dealt with quickly, the time taken to conduct a GC or HPLC analysis is often impractical (Kim *et al.* 2010).

Ultraviolet fluorescence techniques and oil–in–water fluorometers are two methods commonly applied in oil spill analysis to provide a semi-quantitative measure of the hydrocarbons present (Lambert *et al.* 2003; Kim *et al.* 2010). Total petroleum hydrocarbon (TPH) concentration determined by UVF is performed with a spectrophotometer with excitation and emission wavelengths set by the analyst. Oil-in-water fluorometers commonly use a module designed to detect the greatest response of oil components with a broad wavelength range that is fixed by the manufacturer. Oil-in-water fluorometers detect oil between the upper and lower wavelengths. Through calibration techniques they provide a rapid and effective estimation of TPH (UNEP 1992; Lambert *et al.* 2003; Kim *et al.* 2010). Although it often considered that that polycyclic aromatic hydrocarbon (PAH) quantification provides the best correlation with the toxicity of the oil, other studies have shown a stronger relationship between TPH and toxicity (eg. eg. Barron *et al.* 1999; Clarke *et al.* 2001). This previous research suggests that a semi-quantitative measure of TPH concentration may provide an indication of potential toxicity to certain organisms.

In this study, gas chromatography was performed on the 1.00 % WAF of each treatment prior to exposure, to provide a detailed analysis of the treatments. Semi-quantitative methods were performed pre- and post-exposure in each experiment to determine the

percentage total petroleum hydrocarbon concentration remaining following the exposure period. These semi-quantitative analyses are detailed and the results provided in the following chapters.

Quantitative analysis of the 1.00 % w/v water accommodated fraction (WAF) was performed on each treatment by a commercial laboratory, Sydney Environmental and Soil Analysis Laboratory (SESL) (NATA accreditation number 2901). The WAF was produced and stored at the UTS laboratories under the conditions described above and transported to the SESL laboratories at less than 4 °C.

The analyses provided a quantitative measure of the total petroleum hydrocarbons (TPH), carbon chain length fractionation (C_6 to C_9; C_{10} to C_{14}; C_{15} to C_{28}; C_{29} to C_{36}); the mono-cyclic aromatic hydrocarbons, benzene, toluene, ethyl-benzene and xylene (BTEX) and polycyclic aromatic hydrocarbons (PAHs) within each water accommodated fraction petrochemical treatment prior to exposure. The PAHs analysed were naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzanthracene, chrysene, benzo-fluoranthene, benzo-pyrene, indeno-pyrene, dibenzo-anthracene and benzo-perylene. Samples were analysed according to USEPA Standard Methods using Gas Chromatography. Specifically, C_6 to C_9, and benzene, toluene, ethyl-benzene and xylene (BTEX) were analysed directly (no extraction) by purge and trap gas chromatography – mass spectrometry (GC-MS) (USEPA 8020A/ 8000 and USEPA 8260 respectively); C_{10} to C_{36} were extracted with dichloromethane and analysed by GC-flame ion detector (FID) (USEPA 8000); and polycyclic aromatic hydrocarbons were extracted with dichloromethane and analysed by GC-MS (USEPA 8310/ 8270). Only one sample per treatment (n =1) was analysed due to the high cost of the analysis ($160 AUD per sample).

Carbon fractionation results including total TPH and the BTEX analysis are presented in Figures 2.1 and 2.2 for the crude oil treatments and Figures 2.3 and 2.4 for the IFO-380 treatments. Naphthalene and phenanthrene were the two dominant PAHs in most treatments and the concentrations for these are presented in Table 2.1 for both the crude and IFO-380 treatments.

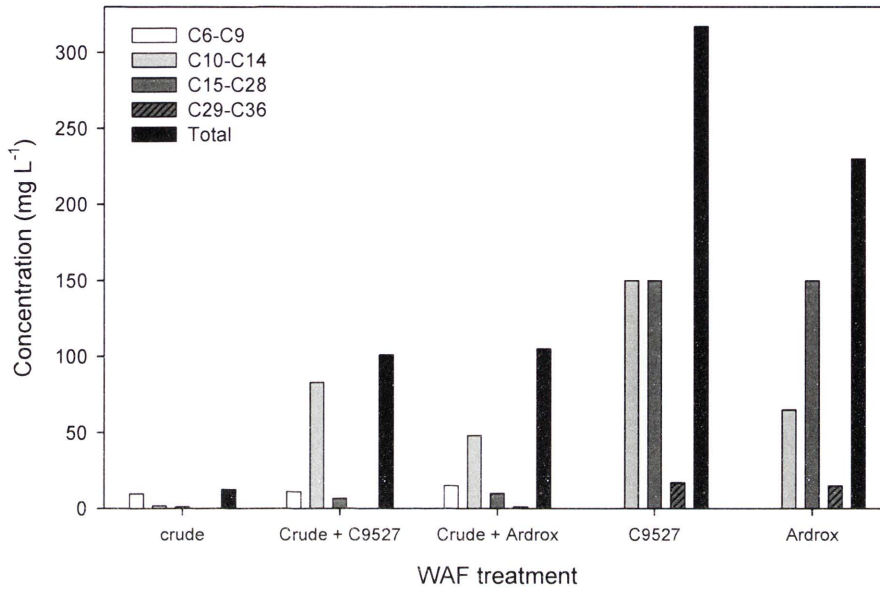

Figure 2.4: Carbon chain length fractionation per treatment and total petroleum hydrocarbon concentration (mg L^{-1}) within the crude, crude + Corexit 9527, Crude + Ardrox, Corexit 9527 alone, Ardrox alone WAF treatments pre-exposure (n = 1).

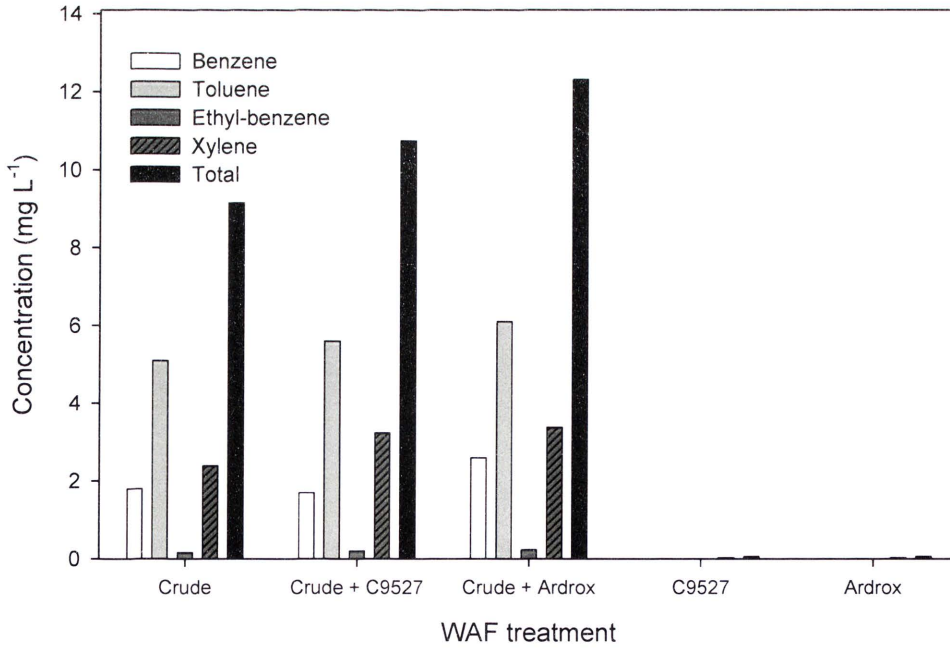

Figure 2.5: BTEX composition (mg L^{-1}) within the crude, crude + Corexit 9527, Crude + Ardrox, Corexit 9527 alone, Ardrox alone WAF treatments pre-exposure (n =1).

The total petroleum hydrocarbon (TPH) concentration (Σ C_6 to C_{36}) for the 1.00 % crude alone water accommodated fraction (WAF), pre-exposure, was 12 mg L^{-1}(Figure 2.4). Compared with the other treatments, this total TPH was quite low. However, it comprised approximately 80 % of highly volatile, light weight hydrocarbons in the C_6 to C_9 range, and this was by far the highest percentage composition of C_6 to C_9 hydrocarbons in any of the treatments, crude or IFO-380.

The addition of the dispersants to the crude oil (Figure 2.4) increased the TPH in the WAF by almost ten-fold. The Corexit 9527 and the Ardrox dispersed oil treatments showed very similar TPH, (101 and 105 mg L^{-1}, respectively) but the two treatments did differ somewhat in their composition. Hydrocarbons within the C_{10}–C_{14} range made up 82 % of the Crude dispersed with Corexit 9527 treatment yet, whilst still representing the greatest proportion of hydrocarbons within the Crude dispersed with Ardrox treatment, only accounted for 45 % of the total composition in that case. The total TPH within the dispersant Corexit 9527 alone and Ardrox alone treatments was high, 317 and 230 mg L^{-1} respectively. Within the Corexit 9527 alone WAF treatment, there was an equal partitioning of hydrocarbons in the C_{10}–C_{14} and C_{15}–C_{28} range, together accounting for 94 % of the total TPH. The Ardrox alone treatment comprised mostly hydrocarbons within the C_{15}–C_{28} range (65 %) and only 28 % within the C_{10}–C_{14} range.

Toluene was the most abundant of the BTEX hydrocarbons within the non-dispersed and dispersed crude treatments (Figure 2.5). The crude alone had a total BTEX concentration of 9 mg L^{-1} with toluene accounting for 54 % (5 mg L^{-1}), followed by xylene (25 %), benzene (19 %) and ethyl-benzene (2 %). The total BTEX concentration was slightly higher in the crude dispersed with Ardrox, 12 mg L^{-1}, than the crude dispersed with Corexit 9527, 11 mg L^{-1}. The composition of the BTEX hydrocarbons, albeit slightly greater in the dispersed crude treatments, was very similar to the non-dispersed crude treatment. By percentage composition for both dispersed crude treatments toluene accounted for 50 to 52 %; xylene for 27 to 30 %; benzene for 16 to 21 %; and ethyl-benzene for 2 % of the total BTEX concentration. The dispersant alone treatments showed only minimal concentrations of the BTEX hydrocarbons but it should be noted that the Practical Quantitation Limit (PQL) was raised due to "foamy

samples" (SESL communication) which may have affected the outcome of this analysis (BTEX) for these two treatments.

For the PAH analysis (Table 2.1) of the non-dispersed and dispersed crude samples, only naphthalene showed concentrations above 5 μg L^{-1} and even these values were low in comparison with the total TPH. The dispersant alone treatments had very low levels of all PAHs.

Table 2.1: PAH constituents naphthalene and phenanthrene concentrations (μg L^{-1}) within the crude, crude + Corexit 9527, Crude + Ardrox, Corexit 9527 alone, Ardrox alone, IFO-380, IFO-380 + Slickgone, IFO-380 + Corexit 9500, Slickgone alone and Corexit 9500 alone WAF pre-exposure. N.B. change of units compared to Figs 2.2, 2.3, 2.4 & 2.5.

Treatment	Naphthalene (μg L^{-1})	Phenanthrene (μg L^{-1})
Crude	82	< 1
Crude + C9527	130	2
Crude + Ardrox	140	2
C9527	< 2	< 2
Ardrox	< 2	< 2
IFO-380	91	13
IFO-380 + Slickgone	66	18
IFO-380 + C9500	320	560
Slickgone	4	< 1
C9500	< 1	< 1

The total TPH within the IFO-380 WAF treatment was low compared with all other treatments 3 mg L^{-1} (Figure 2.6). The majority of hydrocarbons were within the range C_{15}–C_{28}, 43 %, however, the low weight volatile components (C_6–C_9) still accounted for 23 % of the total TPH. Both dispersed IFO-380 treatments increased the TPH within the WAF greatly, with the Corexit 9500 increasing the concentration by almost 200 times above that of the non-dispersed IFO-380 treatment (Figure 2.6). The IFO-380 dispersed with Slickgone had a total TPH of slightly less than 196 mg L^{-1}; this represents an increase in TPH by about 70 times that of the non–dispersed IFO-380 treatment.

Hydrocarbons within the C_{10}–C_{14} range comprised 66 % of the total TPH. Dispersing the IFO-380 with Corexit 9500 resulted in a total TPH of over 522 mg L^{-1} in the 1 % WAF treatment. There were approximately equal proportions of hydrocarbons within the C_{10}–C_{14} and C_{15}–C_{28} ranges, accounting for 90 % of the total TPH. The highly volatile components, C_6–C_9, were all but absent (< 1 % of the total TPH) in both dispersed IFO-380 treatments (Figure 2.6). The total TPH within the Slickgone alone and Corexit 9500 alone treatments were 150 and 167 mg L^{-1} respectively (Figure 2.6).

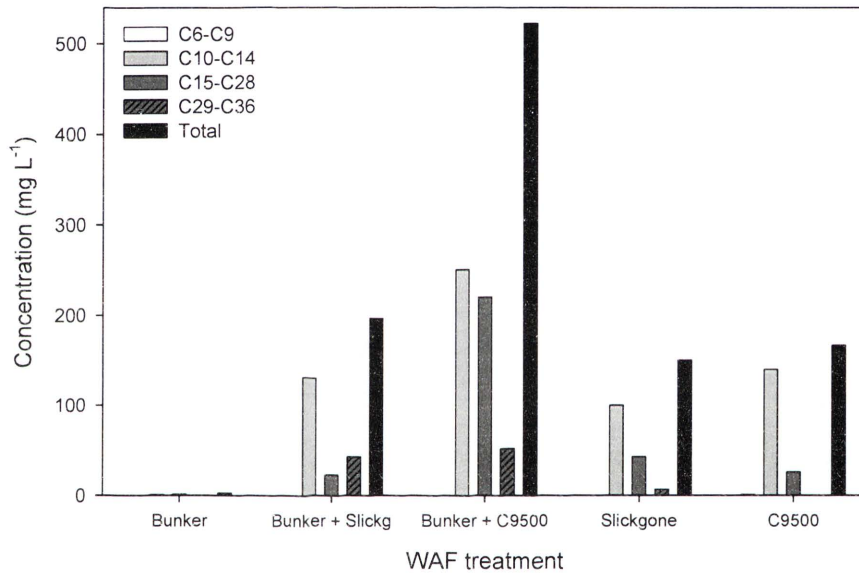

Figure 2.6: Carbon chain length fractionation per treatment and total petroleum hydrocarbon concentration (mg L^{-1}) within the IFO-380, IFO-380 + Slickgone, IFO-380 + Corexit 9500, Slickgone alone and Corexit 9500 alone WAF treatments pre-exposure (n = 1).

Low levels of BTEX hydrocarbons were detected in the IFO-380 treatments compared with the crude oil treatments (Figure 2.7) BTEX hydrocarbons represented about 20 % of the total TPH of the water accommodated fraction of the IFO-380 alone treatment with 82 % of the BTEX total made up of toluene and xylene (Figure 2.7) The concentration of BTEX components within the water accommodated fraction decreased with the addition of the dispersants, but only slightly. For the IFO-380 dispersed with Corexit 9500 this was only a marginal decrease, but for the Slickgone dispersed treatment there was 50 % less BTEX than in the non-dispersed IFO-380, 0.3 mg L^{-1}.

Relative to the total TPH, the BTEX composition was low, less than 1 %, in both dispersed IFO-380 treatments. The dispersant alone treatments were composed of twice as much BTEX components than their respective dispersed IFO-380 treatments. The Corexit 9500 alone treatment was 0.9 mg L^{-1}, the highest concentration of BTEX compounds within any of the IFO-380 treatments (Figure 2.7).

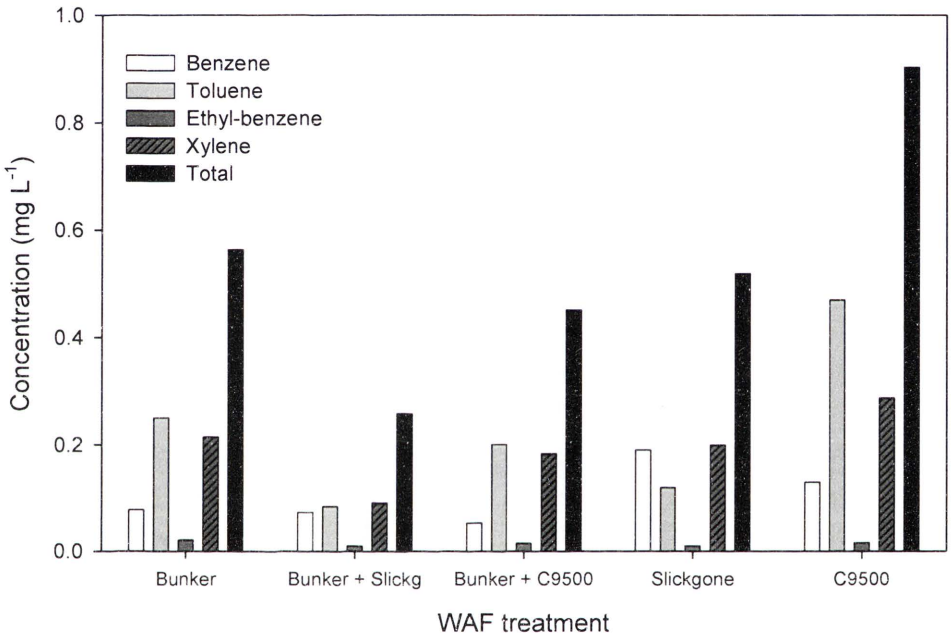

Figure 2.7 BTEX composition (mg L^{-1}) within the IFO-380, IFO-380 + Slickgone, IFO-380 + Corexit 9500, Slickgone alone and Corexit 9500 alone WAF treatments pre-exposure (*n* =1).

Naphthalene and phenanthrene were the two main PAHs detected in the non-dispersed and dispersed IFO-380 treatments (Table 2.1). The IFO-380 alone contained 91 μg L^{-1} of naphthalene and 13 μg L^{-1} of phenanthrene. The IFO-380 dispersed with Slickgone was somewhat similar in PAH composition to the IFO-380 alone treatment. The Corexit 9500 dispersed treatment contained the highest concentration of PAHs in any of the treatments with several of the PAH concentrations detected above 100 μg L^{-1}. Naphthalene and phenanthrene showed the highest concentrations (Table 2.1). Other PAHs at relatively high concentrations (data not shown) were pyrene (220 μg L^{-1}), chrysene (160 μg L^{-1}) and benzanthracene (110 μg L^{-1}). The PQL was raised in the PAH

analysis due to the Corexit 9500 sample's "high concentration of analytes in the sample". All other PAHs analysed had a concentration of < 100 μg L^{-1}, except for benzo-fluoranthene which had a concentration of < 200 μg L^{-1}. The dispersant alone treatments had low PAH levels.

Semi – quantitative methods of total petroleum hydrocarbon (TPH) concentrations were performed at the UTS laboratories for both *in situ* and laboratory experiments. These were conducted pre and post exposure for each concentration within each treatment (n = 3 per concentration) and are described and presented in the relevant chapters.

2.4 Statistical Analysis

Effective quantum yield of PSII

The effective quantum yield data ($\Delta F/F_m'$) were expressed and analysed as a change from the baseline (pre-petrochemical exposure) per replicate and as a change compared with the control mean at each time. This technique was considered important as slight variations in the pre-exposure measurements may lead to invalid detections of significant differences in the post –test measurements (Bonate 2000; Muller *et al.* 2008). This procedure also accounted for variation in the control due to natural incidences overtime (eg. fluctuations in light attenuation). This technique reduces potential bias and provides greater confidence when accepting or rejecting the null hypothesis (Bonate 2000). This meant that the control mean was zero at each timepoint, with a positive change representing an increase in $\Delta F/F_m'$ (hormetic response) and a negative change representing a reduction in $\Delta F/F_m'$. Presenting the data as a change measurement, however, can reduce the simplicity of visual comparisons. This is particularly evident with measurements such as units of $\Delta F/F_m'$ (eg. a decrease of 0.2 units of Δ $\Delta F/F_m'$ means little to most analysts). In the following chapters, the minimum y-axis value has been set at -0.6 units (0.6 units $\Delta F/F_m'$ being the approximate $\Delta F/F_m'$ of healthy seagrass used in this study) to give an approximation of the maximum possible negative response.

All data were analysed using statistical software (SSPS® v.17.0) and graphed using SigmaPlot (v.10). Data were tested for normality using Kolmogorov–Smirnov and homogeneity of variance by assessing plots of the residuals and by Levene's test for Homogeneity of variance. Data were square root transformed when these assumptions were not met.

Effective quantum yield data of each individual treatment were analysed by repeated measures two-way analysis of variance (rmANOVA). Statistical analyses were not performed between the different treatments (for example, oil alone was not compared to dispersed oil). This was because of the different times when the different treatments were conducted, and because there was no replication of each treatment (i.e. each treatment was conducted only once in each season). The rmANOVAs conducted on each individual treatment (for example Tapis crude oil alone) were used to determine if significant differences were present over time, between WAF concentrations and the control, and the interaction between time and concentration. Separate rmANOVAs were conducted for the exposure and recovery periods as there were different conditions between these two components within each experiment (see Chapters Three and Four for experimental design description). Significant effects were further investigated using one-way ANOVAs between concentrations at each time. Where a significant difference was detected, Tukey's *post hoc* Honestly Significant Difference (HSD) tests were conducted to determine which concentrations were significantly different. Pairwise comparisons were used to determine which timepoints were different to each other

3 Impacts of Petrochemicals *In Situ*

3.1 Introduction

In situ experiments provide the most realistic measure of response from organisms when conducting manipulative experiments. When attempting to recreate an oil spill event the number and nature of environmental variables in an experiment is complex and highly interdependent. Factors which occur in the field may not be able to be replicated within a laboratory environment (Clark & Noles 1994; Hemming & Duarte 2000). Microbial breakdown of oil occurs once oil enters the environment, as does the incorporation of oil into the sediments (Leahy & Colwell 1990; Page *et al.* 1999; Fingas 2001). Both of these elements, microbial activity and sediment, are often severely reduced or absent in laboratory studies, resulting in an alteration of the fate of petrochemicals and thereby toxicity to an organism. Furthermore, fluctuating cloud cover and rippling wave surfaces, produce fluctuating light intensities in the water column altering the photodegradation of oils (Hemminga & Duarte 2000). Couple this, with scattering of light within the canopy of a seagrass meadow due to movement of the seagrass blades which not only affects the rate of oil breakdown but also the photosynthesis of the seagrass, and it is obvious that laboratory experiments are often lacking in terms of representing natural conditions. Because of this laboratory results are often not as useful as field results when recommendations for field applications are required (Thorhaug *et al.* 1986).

Experiments assessing the response of subtidal seagrass to petrochemicals have been conducted with varying outcomes ranging from whether there is an affect or not, and the level to which this effect was observed (eg. Baca & Getter 1984; Thorhaug *et al.* 1986; Ralh & Burchett 1998a; Macinnis & Ralph 2003b). Previous research suggests that amongst seagrass species there is a wide diversity of responses to petrochemicals (Thorhaug *et al.* 1986; 1988) and studies investigating the effects of chemically dispersed oil to subtidal species have concluded vastly different findings. Baca and

Getter (1984) and Macinnis and Ralph (2003b) found the addition of chemical dispersants to oil had less of an effect to *Z. capricorni* than non-dispersed oil, Hatcher and Larkum (1982) found the complete opposite in that the dispersed oil had a greater impact than the non-dispersed oil on *Posidonia australis*; whilst Ralph & Burchett (1998a) found only minimal impact on *H. ovalis* from either dispersed or non-dispersed oil. Apart from the different species investigated, other factors that were different between the studies include different petrochemicals and different methodologies. However, another major factor that is often not investigated in seagrass response to oils and dispersed oils is the influence of temperature and location.

Marine macrophytes tend to have slower growth rates during winter, than during summer. Suggested reasons for seasonal differences include differences in photoperiod, irradiance and water temperature (Larkum *et al.* 1984; Kerr & Strother 1990; Masini *et al.* 1997). Furthermore, tropical species tend to have faster growth rates and adapt differently to change than temperate species (Waycott *et al.* 2005). Hatcher and Larkum's (1982) microcosm study was conducted in a 20-25 °C holding room between March and August; Macinnis and Ralph did not detail the temperature in their (2003) paper but it is described in Macinnis (2000, PhD thesis) as a water temperature of 18 °C; whereas the Thorhaug *et al.* (1986) study was conducted in both summer and winter, in the tropics, unlike the former studies which were conducted in temperate locations. The growth rate of a plant is likely to affect it's response to an external stress (Waycott *et al.* 2005). Lyngby and Brix (1982) suggested seasonal differences in metal concentrations in seagrass blades was due to differences in seagrass productivity and growth rates, whilst Brun *et al.* (2002) found clear differences in the response of *Zostera nolteri* with exposure to ammonia in different seasons. It is likely that similar differences occur with seagrass response to petrochemicals yet it remains to be investigated.

Oils also behave differently at different temperatures with higher temperature making the oil less viscous, and increasing the solubility of hydrocarbons into the water column (Burridge & Shir 1988; Nemr 2000; Singer *et al.* 2000). Oils have been shown to lose the light volatile fractions more rapidly at higher temperatures than at lower temperatures (Nemr 2000). As these lighter volatile fractions have been shown to be the

most toxic, seasonal differences in the toxicity of the oil are likely to occur (Nemr 2000). For these reasons, it is suggested that the impacts to organisms from petrochemicals should be different at different temperatures.

The major aims of this chapter were to determine the effects of non-dispersed oil and dispersed oil on *Z. capricorni* (and *Z. muelleri*) *in situ*. The specific objectives were to: 1) determine the effect of petrochemical treatments at different concentrations; 2) determine the nature of response in relation the level of impact, time of impact and recovery; 3) determine the response during summer and winter experiments and; 4) determine the response at different locations.

3.2 Methods

Experimental Design

Fifteen clear perspex cylinders (300 mm high and 125 mm diameter, 12 L volume) were placed in *Z. capricorni* meadows at Bonna Point, and *Z. muelleri* meadows in Corio Bay. The cylinders were pushed into the sediment to approximately 5 cm deep. The cylinders were secured with four pegs attached by ropes to the external cylinder wall. Two Perspex tubes (10 mm diameter) were attached to the external cylinder wall to house timber stakes pushed in to the sediment to further increase stability particularly during rough weather conditions. Specially designed perspex lids sealed the cylinders and were held in position with rubber bands.

Bilge pumps (Rule 12 Volt, USA) were used to provide aeration within the chambers and were attached to the underside of the chamber lids. Power to the pumps was via sealed lead acid (SLA) batteries (DiaMec 18.0 Amp 12 Volt, Hong Kong) contained within specially designed submersible housings. The set up consisted of three separate housings each with five bilge pumps attached to support the 15 chambers used in the experimental design. SLA batteries were charged within the laboratories the night before the experimental exposure day and were switched on at the commencement of

the exposure period. Flexible common 1 mm plastic mesh was secured over the intake end of each bilge pump to reduce plant and other materials entering the pump and blocking the propeller, and causing the pump to burn out.

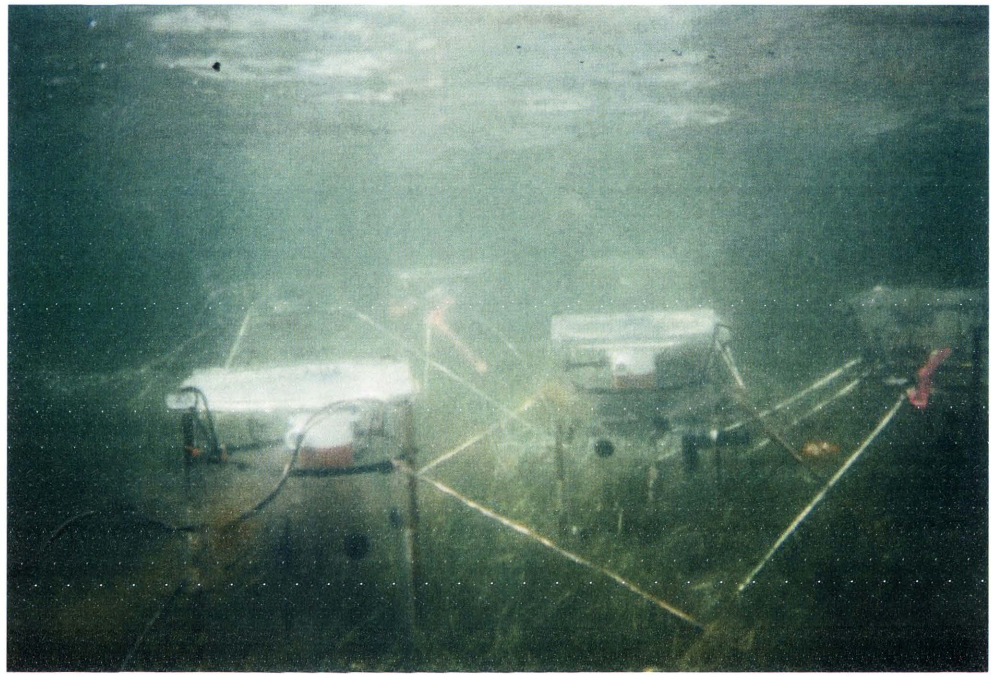

Figure 3.1: Photo of mesocosms in the seagrass meadows of Corio Bay, Port Phillip Bay, Victoria.

Inside the exposure cylinder, healthy seagrass blades were held in position by specially designed leaf clips supported by plastic stakes (Figure 3.2). This allowed the blades to retain their natural orientation and enabled measurements to be taken from the same position on the leaf blade throughout the exposure day. A 2 mm optic fibre (PolyOptic, Australia) was held in a 45° position relative to the leaf clip and extended outside the cylinder wall through a rubber blanking plug. This enabled remote fluorescence measurements to be taken from outside of the cylinder. The cylinders and leaf clips were set up the day before the exposure day, at low tide, and left overnight.

For the Bonna Point experiments, petrochemical treatments consisted of the water accommodated fraction (WAF) of (i) Tapis crude oil, (ii) Tapis crude oil with Corexit 9527, (iii) Corexit 9527 alone, (iv) IFO-380, (v) IFO-380 with Slickgone and (vi)

Slickgone alone and experiments were conducted in summer (December – February) and winter (June – August). For the Corio Bay experiments, petrochemical treatments consisted of the WAF of (i) Tapis crude oil and (ii) Tapis crude oil with Corexit 9527 and experiments were conducted in summer only. The different treatments were exposed to the seagrass at different times.

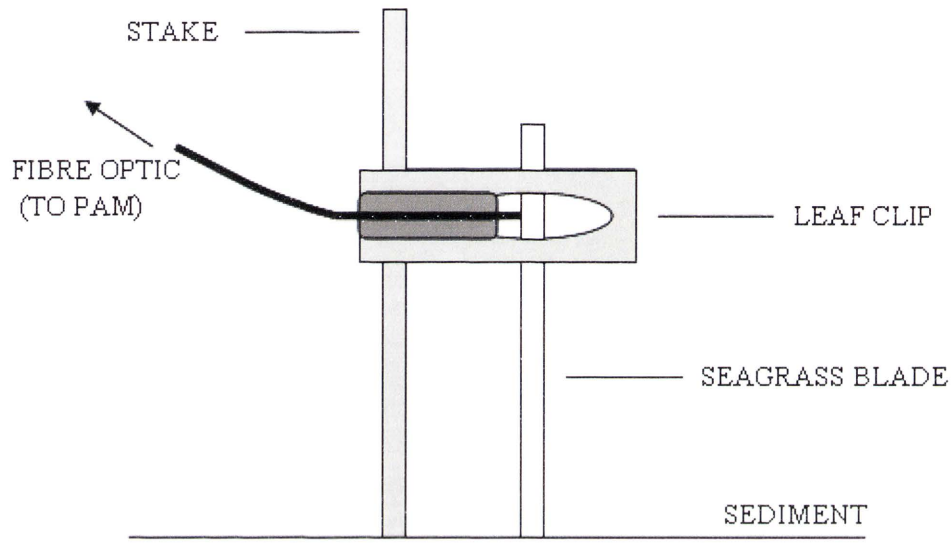

Figure 3.2: Schematic diagram showing the positioning of the seagrass blade in the leaf clip and the fibre optic (not to scale).

This was due to the logistical impracticality of conducting all treatments at the same time (for example, conducting all treatments simultaneously required 90 cylinders). For each experiment, five concentrations (0.00, 0.05, 0.10, 0.20 and 0.40 % WAF) of the specific petrochemical were added with three replicates per concentration. The WAF was added to the cylinders in different volumes specific to the desired concentration (i.e. half the cylinder volume would give half the initial WAF concentration). The WAF was poured into a plastic bag which was then loosely tied. The bag was gently pushed in to the top of the cylinder, the lid placed on the cylinder and the bag extracted through a blanking plug in the chamber wall.

An initial effective quantum yield of PSII measurement was taken prior to the petrochemical exposure and then every two hours until 1700 hours. At 1700 hours, the chamber lids were removed and the ramet of seagrasss used for the $\Delta F/ F_m'$ measurements was tagged using Forrester's Tape. Several seagrass leafblades (those not tagged) were collected from within the chambers for later pigment analysis and a water sample was also collected for chemical analysis. The cylinders were then removed to allow replenishment of 'fresh' seawater to the seagrass. Seagrass recovery was monitored by performing a single $\Delta F/ F_m'$ measurement on each tagged ramet at approximately 0700 hours for the following four days. Seagrass blades were also collected at the end of the recovery period (96 hours following the initial exposure) for a second series of pigment analyses. The recovery period was incorporated to assess seagrass response once the petrochemicals were removed. Primarily, the recovery period sought to observe whether impacts during the exposure day were short-lived or of a more chronic nature.

There were two control treatments in the *in situ* experiments: (i) three replicate external controls, where measurements were performed on seagrass not within mesocosms and not exposed to the WAF treatments; and (ii) three replicate internal controls within the mesocosm, with no WAF treatment added. These controls were designed to address, and account for, possible effects of the chambers themselves.

Chemical Analysis

Ultraviolet fluorescence (UVF) analyses were conducted at the University of Technology, Sydney, as a semi quantitative measure of loss of total petroleum hydrocarbon (TPH) over the exposure period. UVF analyses were conducted on samples prior to addition to the chambers, and at the end of the exposure period for all treatments ($n = 3$) in both summer and winter. Water samples from each chamber were siphoned into 2.5 L amber glass bottles at the end of the exposure period. These were transported to the UTS laboratory, preserved with 10 mL methanol and stored in the dark at 4° C until analysis within 28 days (APHA 1995).

Samples were extracted through solid phase extraction (SPE) cartridges (Oasis HLB 6cc, Waters, USA) using a Supelco VisiPrep Extraction Manifold (USA). The samples were adjusted to a pH of 2 by adding 2 ml of hydrochloric acid. Cartridges were pre-treated with two, 5 ml aliquots of methanol, followed by two, 5 ml aliquots of milliQ water. Samples were passed through the cartridges at approximately 30 ml per minute and eluted with two, 5 ml aliquots of *n*-hexane (low aromatic hydrocarbons- Sigma Aldrich). Sample bottles were further rinsed with two, 5-ml aliquots of *n*-hexane to ensure complete hydrocarbon removal. Samples were then made to volume in 25 ml volumetric flasks with *n*-hexane.

The WAF samples were analysed for TPH using ultraviolet fluorescence (UVF) with a spectrophotometer (Varian Cary Eclipse, USA). Semi-quantitative calibration curves were determined for each treatment. This produced measurements in relative oil and dispersed oil units (ppm) but were respective to each treatment (eg. relative Tapis crude units, relative Tapis crude and Corexit 9527 units). As these values were not comparable between the different treatments, all values were expressed as the percentage remaining over the exposure day for each treatment ((post-exposure concentration/ pre–exposure concentration) x 100). Thereby, the UVF data enabled a treatment comparison of loss of TPH over the exposure period, whereas the GCMS data (Chapter Two) enabled a quantitative determination of TPH, BTEX and PAH within the treatments. The TPH concentration of the dispersant alone treatments were not determined in these experiments (via UVF in this chapter) due to a lack of linearity in the calibration curves.

The spectrophotometer settings were for crude oils: excitation wavelength, 310 nm; emission wavelength, 360 nm; IFO-380: excitation wavelength, 270 nm; emission wavelength 330 nm; and excitation slits were set at 10 nm. Quality was monitored with the use of seawater and *n*-hexane controls which were stored, extracted, eluted and analysed in the same manner as the WAF samples. All glassware were soaked with Pyroneg©, rinsed with distilled water, rinsed with milliQ water, and finished with three *n*-hexane (low aromatic hydrocarbons, Sigma Aldrich) rinses.

Photosynthetic pigment analysis

Chlorophyll *a* pigment analyses were performed on seagrass at the end of the exposure day and at the conclusion of the recovery period (96 hours). Seagrass blades were gently scraped free of epiphytes, dried on paper towel and weighed on an electronic balance. Samples were initially placed in two ml of acetone in 15 ml amber glass bottles and refrigerated for an hour. Samples were then ground in 10 ml of 90 % acetone with a mortar and pestle and a pinch of acid-washed sand (Denison 1994). Several drops of $MgCO_3$ were added and the samples were centrifuged and refrigerated (4 °C) for 1 day prior to analysis. Absorbance was measured at 480, 647 and 664 nm, and all readings were corrected for turbidity scattering by subtracting the 750 nm absorbance. A spectrophotometer (LKB Introspect II UV/Vis, model 4050) with a spectral resolution of 1.00 nm was used for the absorbance measurements.

Statistical analysis

See General Methods Section (Chapter Two, p.52) for more detail.

Analysis of variances (ANOVAs) were conducted to determine statistical differences in the $\Delta F/ F_m'$ between the internal and external controls. Significant differences were detected in some treatments, at some times (eg. Crude alone water accommodated fraction treatment in summer, four hours exposure ($F = 13.327$, $p = 0.022$)). As significant differences exist between the external and internal control at some times in some of the treatments, it was deemed necessary to correct for these chamber effects as they were considered an experimental artefact. A percentage difference of the mean of the external control and the mean of the internal control at each time within each treatment were calculated. This percentage value was then used to calculate the $\Delta F/ F_m'$ values of the remaining WAF treatments and these corrected values were used.

3.3 Results

3.3.1 Tapis crude oil: non-dispersed, dispersed and dispersant alone

Petrochemical analysis (UVF)

The results of the semi-quantitative measure of loss of total petroleum hydrocarbon (TPH) over the exposure period, by ultraviolet fluorescence (UVF), are expressed as percentage loss and displayed in Table 3.1 . All concentrations within the non-dispersed and dispersed Tapis crude treatments decreased significantly over the exposure period (Table 3.1).All non-dispersed Tapis crude treatments decreased to below detection limits following the ten hour exposure period. Only the higher WAF treatments of the Corexit 9527 dispersed crude displayed any measurable concentrations at the end of the exposure period (Table 3.1). All Botany Bay treatments exhibited a significant decline in TPH in summer ($P < 0.01$) and in winter ($P < 0.05$) and there was a higher percentage TPH recovered in winter compared with summer. In the Corio Bay experiments, TPH in the crude dispersed with Corexit 9527 significantly declined from the pre-exposure value ($P < 0.01$).

Table 3.1: Percent TPH remaining following 10 hour field exposure of the water accommodated fraction (WAF) of Tapis crude oil alone, and Tapis crude oil dispersed with Corexit 9527 in summer and winter in Botany Bay, New South Wales, and Corio Bay, Victoria.

Treatment			WAF concentration (% remaining post-exposure)			
	Site	Season	0.05 %	0.10 %	0.20 %	0.40 %
Tapis	Botany Bay	Summer	nd	Nd	nd	Nd
	Botany Bay	Winter	nd	Nd	nd	Nd
	Corio Bay	Summer	nd	Nd	nd	Nd
Tapis + C9527	Botany Bay	Summer	nd	Nd	<10 %	14 %
	Botany Bay	Winter	nd	< 10 %	32 %	28 %
	Corio Bay	Summer	nd	nd	< 10 %	15 %

Chlorophyll *a* fluorescence

Repeated measures analyses of variances (ANOVAs) were conducted to detect impacts to the seagrasses from each individual petrochemical treatment (e.g. Tapis crude oil alone), over the exposure day and recovery days for the summer and winter experimental periods. Where there were significant differences, one-way ANOVA's were performed to determine the 'simple effects' of when and how these differences occurred (Quinn & Keough 2002). These one-way ANOVA's were conducted at each time during the exposure and recovery days and are presented inTable 3.3 (Botany Bay, Sydney) and Table 3.5 (Corio Bay, Melbourne) for the crude treatments and Table 3.10 for the IFO-380 treatments. Tukey's post hoc comparisons were determined to detect differences between the WAF treatments and the control and the outcomes are described in the following text. Figures 3.2 to 3.15 display the change in effective quantum yield over the exposure and recovery days for *Z. capricorni* and *Z. muelleri*. Only two experiments (those conducted on *Z. Muelleri*; Figures 3.8 & 3.9) were undertaken at the second location, Corio Bay, Victoria (VIC). All other experiments were conducted in Botany Bay, New South Wales (NSW).

Minor impacts were detected in the photosynthetic health of *Z. capricorni* exposed to the crude alone WAF treatment (Figure 3.3) over the exposure and recovery periods in summer. Most WAF concentrations changed less than 0.1 units in $\Delta F/ F_m'$ from the control at all times (Figure 3.3). A significant concentration effect ($P < 0.05$; Table 3.2) was due to the 0.05 % WAF being significantly elevated above the 0.40 % WAF treatment at six and eight hour's exposure ($P< 0.05$; Table 3.3); but at no time during the exposure and recovery periods did any of the treatments differ from the control (Table 3.2).

In the winter experiment of the crude alone WAF treatment (Figure 3.4) there was a significant concentration effect ($P <0.01$) with the $\Delta F/ F_m'$ of the seagrass exposed to the 0.20 % and 0.40 % WAF concentrations significantly lower than the control, 0.05 and 0.10 % WAF concentrations. One way ANOVAs detected differences after six and

eight hours exposure ($P < 0.05$). Tukey's post hoc test did not detect differences at six hours, but at eight hours, both the 0.20 % and 0.40 % WAF concentrations were significantly lower than the control. There was a significant time effect over the recovery period (Table 3.2) with the $\Delta F/ F_m'$ of the 0.20 % and 0.40 % WAF significantly lower than the control and 0.05 % concentration ($P < 0.05$) at 48 hours.

The crude dispersed with Corexit 9527 treatment in summer (Figure 3.5) led to a significant interaction (time x concentration) effect on the seagrass during the exposure period ($P < 0.05$; Table 3.2). The 0.20 and 0.40 % WAF concentrations elicited a significantly decreased $\Delta F/ F_m'$ response compared with all other concentrations including the control ($P < 0.001$) up to four hours after exposure (Table 3.3). This negative impact to the seagrass was short – lived and no other concentrations differed significantly to the control throughout the remainder of the exposure period. Over the recovery period the $\Delta F/ F_m'$ of the 0.40 % WAF was significantly lower than that of the control overall ($P < 0.05$; Table 3.2). Differences were detected at 24 hours (Table 3.3) with the 0.40 % WAF significantly lower than both the control and the 0.10 % treatment ($P < 0.05$). Similar to the summer experiment, the crude dispersed with Corexit 9527 WAF treatment in winter (Figure 3.6) showed a short-lived impact on the seagrass during the exposure day. At four hours exposure, the 0.40 % WAF was significantly less than both the control and the 0.05 % WAF treatment ($P < 0.01$; Table 3.3). No further impacts were detected in the $\Delta F/ F_m'$ of the seagrass over the remainder of the exposure day or during any of the recovery days (Table 3.2).

There was no detectable impact to *Z. capricorni* with exposure to the Corexit 9527 alone treatment in summer (Figure 3.7), with no concentrations causing a significant decrease in the $\Delta F/ Fm'$ of the seagrass at any time during the exposure or recovery periods. A significant interaction effect in the Corexit 9527 alone WAF treatment in winter occurred (Figure 3.8, Table 3.2). While the 0.40 and 0.20 % WAF concentrations were significantly lower than the control overall ($P < 0.05$), one way ANOVAs (Table 3.3) detected differences only at four hours exposure ($P < 0.01$). No further differences were detected during the remainder of the exposure or recovery days (Figure 3.8; Table 3.2).

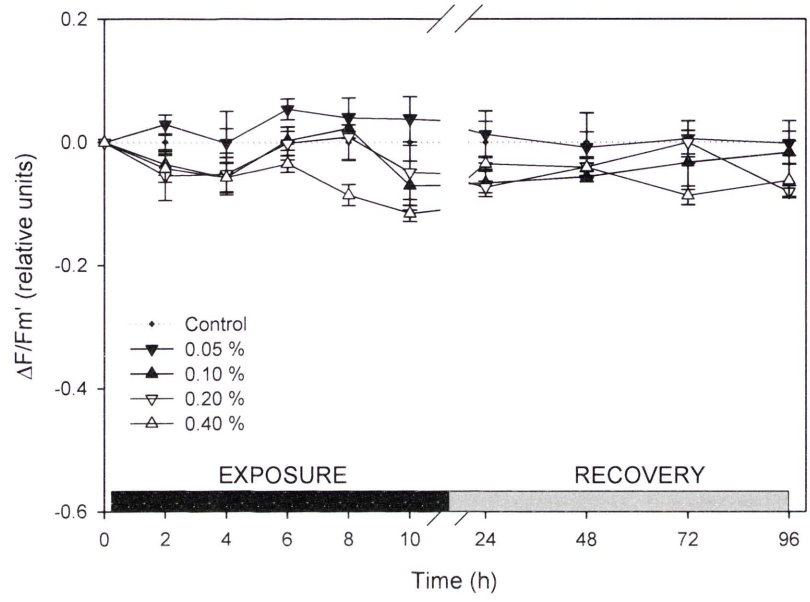

Figure 3.3: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of Tapis crude oil over the exposure and recovery days in summer. Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n = 3).

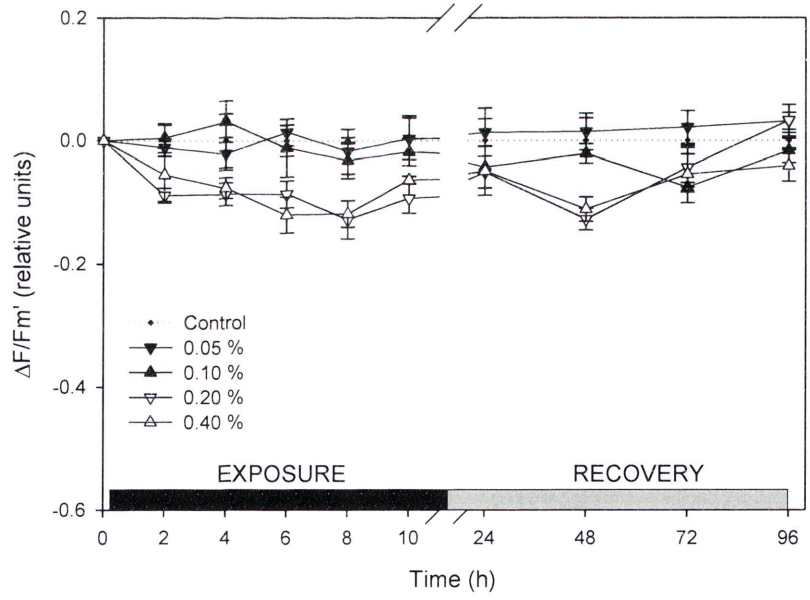

Figure 3.4: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of Tapis crude oil over the exposure and recovery days in winter. Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n =3).

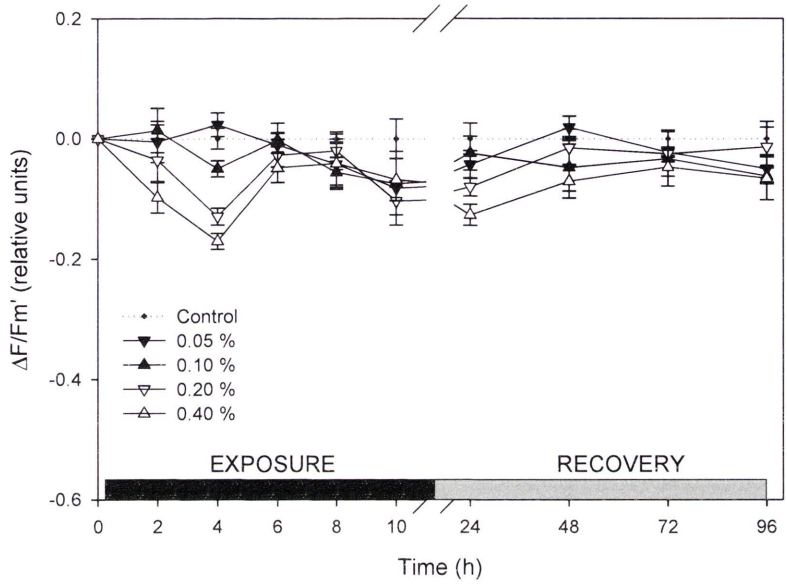

Figure 3.5: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of Tapis crude oil and C9527 over the exposure and recovery days in summer. Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n =3).

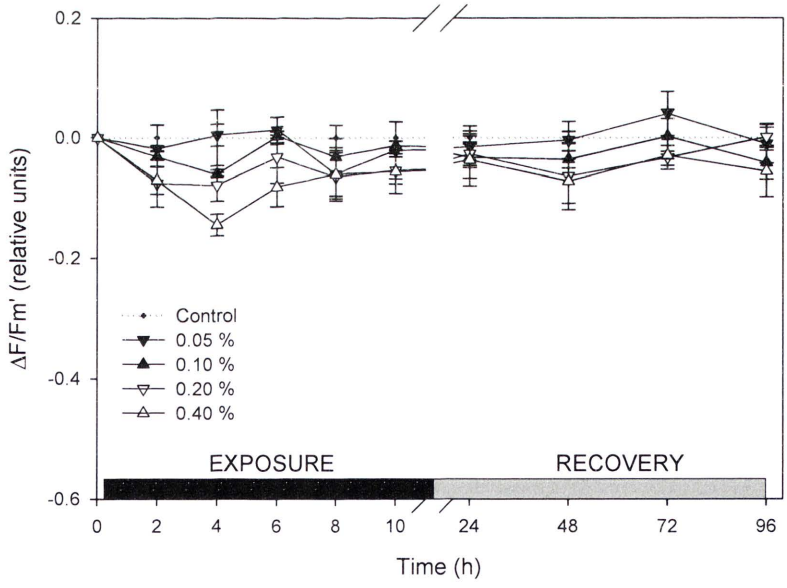

Figure 3.6: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of Tapis crude oil and C9527 over the exposure and recovery days in winter. Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n =3).

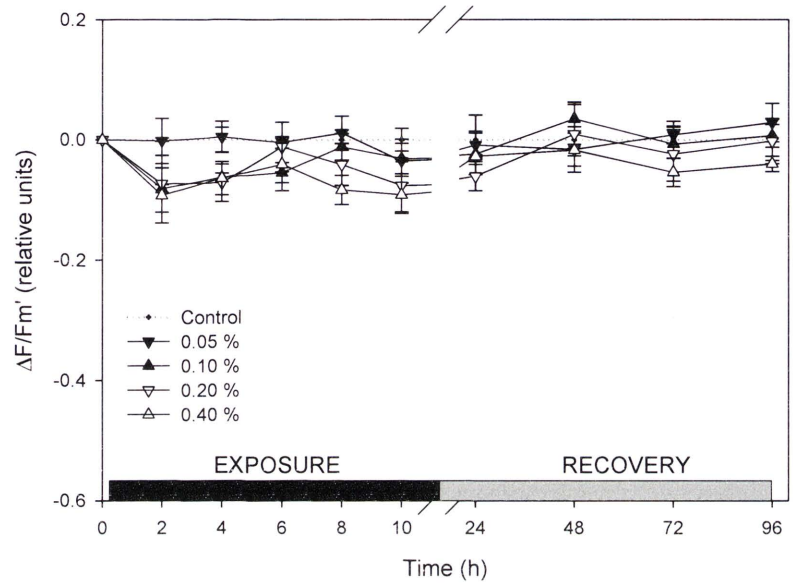

Figure 3.7: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of C9527 over the exposure and recovery days in summer. Percent change from control. Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n =3).

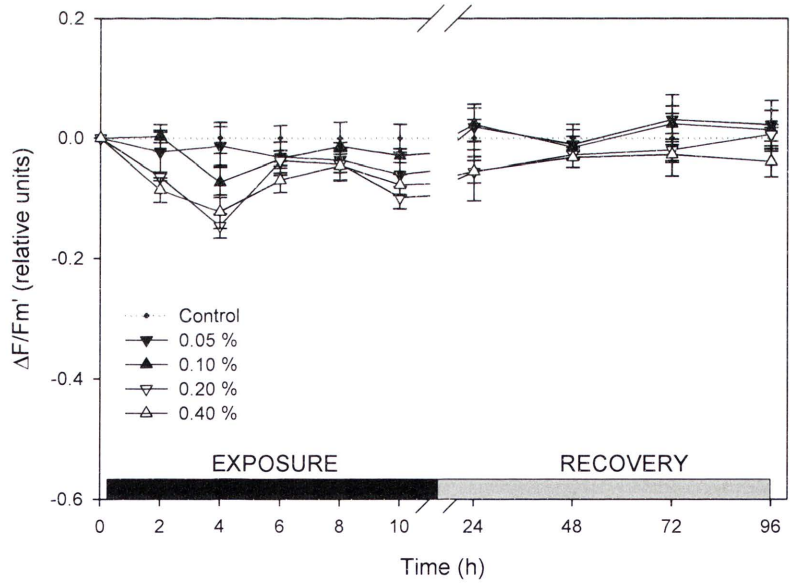

Figure 3.8: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of C 9527 over the exposure and recovery days in winter. Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n =3).

In the experiments conducted at the southern location, in Victoria, there was a visible and significant increase in $\Delta F/ F_m'$ of the seagrass, *Z. muelleri*, in both the non-dispersed and dispersed WAF of Tapis crude treatments (Figure 3.9, Figure 3.10). In the crude alone treatment there were significant differences for both the time and concentration effect (Figure 3.9; Table 3.4) during the exposure day. After eight hours exposure the 0.05, 0.10 and 0.40 % WAF concentrations were significantly elevated above the control ($P < 0.01$; Table 3.5), with the 0.05 % WAF remaining significantly elevated above the control at ten hours ($P < 0.05$; Table 3.5). During the recovery days there was a significant interaction and time effect (Table 3.4). At 24 hours, the control was significantly lower than the $\Delta F/ F_m'$ of the 0.05 and 0.20 % WAF concentrations (Table 3.5). There was a gradual decrease in $\Delta F/ F_m'$ over the next two days. At 96 hours the 0.05 % WAF was significantly elevated above the 0.20 % WAF, but no concentration differed to the control after the first recovery day (Table 3.5).

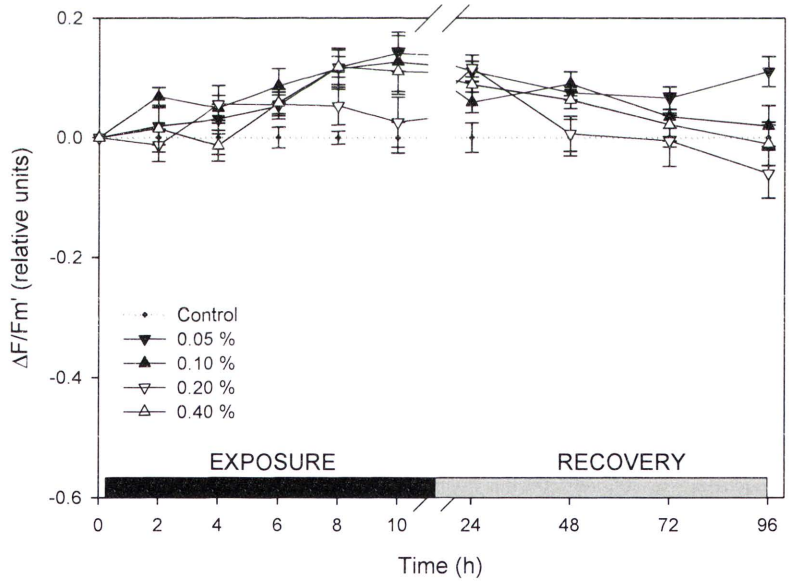

Figure 3.9: Change in effective quantum yield of *Z. muelleri* exposed to the water accommodated fraction (WAF) of Tapis crude oil over the exposure and recovery days in summer in Corio Bay (VIC). Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n =3).

In the dispersed Tapis crude WAF treatment (Corio Bay), all treatments were elevated above the control for most of the exposure day and the first recovery day (Figure 3.10). There was a significant main effect of time and concentration over the exposure day (Table 3.4). The time effect ($P < 0.01$; Table 3.4) occurred with the elevation in $\Delta F/ F_m'$ of all WAF treatments up to the final measurement, ten hours ($P < 0.01$; Table 3.4). At eight and ten hours exposure, the 0.40 % WAF was significantly greater than the control, whilst at ten hours, the 0.10 % was also greater than the control (Table 3.5). The time effect ($P < 0.01$; Table 3.4) during the recovery was due to the high $\Delta F/ F_m'$ in the 24 hour measurements relative to the decreases at 72 and 96 hours (Figure 3.10).

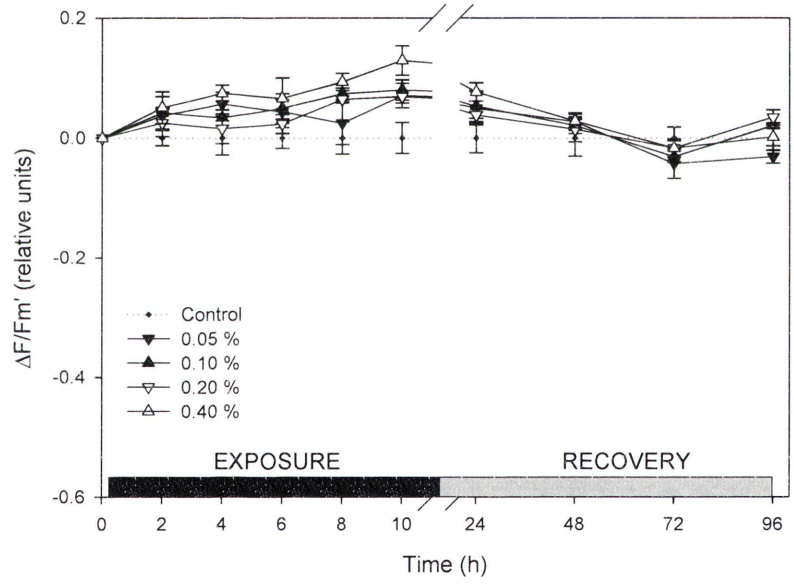

Figure 3.10: Change in effective quantum yield of *Z. muelleri* exposed to the water accommodated fraction (WAF) of Tapis crude oil and Corexit© 9527 over the exposure and recovery days in summer in Corio Bay (VIC). Time zero is pre-petrochemical exposure. Averages ± standard error of the mean are shown (n =3).

Photosynthetic pigment analysis

Few differences were detected in the chlorophyll *a* concentration of *Z. capricorni* at 10 hours and 96 hours for any of the petrochemical treatments (Table 3.6). A significant difference was detected in the chlorophyll *a* concentration at 96 hours in the summer

Corexit 9527 alone water accommodated fraction (WAF) treatment (Table 3.6), where the two largest treatments, 0.20 and 0.40 % WAF were significantly lower in chlorophyll *a* concentration than all other treatments and controls.

There were no significant differences between the chlorophyll a pigment concentrations of *Z. muelleri* after ten hours exposure to the non-dispersed or dispersed crude oil (Table 3.7). There were significant differences detected at 96 hours in the crude alone treatment. The chlorophyll *a* pigment concentration in the 0.05 % WAF treatment was significantly elevated above the 0.20 % WAF treatment (Table 3.7). There were no other significant differences at 96 hours for either dispersed or non-dispersed crude oil.

Table 3.2: Repeated measures ANOVA for the $\Delta F/F_m'$ data of *Z. capricorni* exposed to the different concentrations of a) Tapis crude oil alone, b) Tapis crude oil + Corexit 9527 (C9527) and c) Corexit 9527 alone treatments in summer and winter in Botany Bay, New South Wales. Degrees of freedom for interaction were exposure = 20.8, recovery = 22; for time effect exposure = 6, recovery = 7; and for concentration effect exposure = 4, recovery = 4. Bold denotes significant difference at P = 0.05.

	Exposure		Recovery	
Treatment	*F*	*P*	*F*	*P*
Summer				
a. Tapis				
Concentration	3.229	**0.040**	3.163	0.064
Time	2.598	0.128	1.696	0.244
Concentration x Time	0.899	0.580	1.900	0.094
b. Tapis + C9527				
Concentration	2.721	**0.015**	3.810	**0.039**
Time	6.657	**0.016**	1.953	0.200
Concentration x Time	2.536	0.106	1.417	0.233
c. C9527				
Concentration	1.894	0.188	0.430	0.784
Time	3.859	0.064	2.823	0.107
Concentration x Time	1.888	0.083	0.790	0.656
Winter				
a. Tapis				
Concentration	7.935	**0.004**	2.977	0.072
Time	1.031	0.454	4.127	**0.016**
Concentration x Time	0.549	0.889	1.429	0.315
b. Tapis + C9527				
Concentration	2.993	0.073	0.862	0.519
Time	4.197	**0.048**	1.604	0.263
Concentration x Time	1.640	0.139	0.577	0.837
c. C9527				
Concentration	4.352	**0.027**	0.962	0.469
Time	19.944	**0.001**	2.812	0.108
Concentration x Time	3.688	**0.003**	0.925	0.540

Table 3.3: One way analysis of variance (ANOVA) of $\Delta F/ F'_m$ of *Z. capricorni* exposed to the Tapis crude oil alone, Tapis crude oil + Corexit 9527 (C9527) and Corexit 9527 (C9527) alone treatments in summer and winter at each sampling time in Botany Bay, New South Wales. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text. nc denotes ANOVA not calculated (no significant difference in the rmANOVA - Table 3.1).

Treatment		Exposure					Recovery			
		2	4	6	8	10	24	48	72	96
Summer										
Tapis	*F*	3.584	0.847	3.509	3.803	2.570	nc	nc	nc	nc
	P	**0.046**	0.527	**0.049**	**0.039**	0.103	nc	nc	nc	nc
Tapis +	*F*	2.077	29.103	0.745	0.514	1.156	5.040	1.807	1.754	1.144
C9527	*P*	0.159	**0.000**	0.583	0.728	0.386	**0.017**	0.204	0.215	0.391
C9527	*F*	nc	Nc	nc	Nc	nc	nc	nc	nc	nc
	P	nc	Nc	nc	Nc	nc	nc	nc	nc	nc
Winter										
Tapis	*F*	3.402	2.926	3.850	6.458	2.406	0.581	7.075	1.746	2.423
	P	0.053	0.077	**0.038**	**0.008**	0.119	0.683	**0.006**	0.216	0.117
Tapis +	*F*	1.972	11.321	1.973	0.650	0.830	nc	nc	nc	nc
C9527	*P*	0.175	**0.001**	0.175	0.640	0.536	nc	nc	nc	nc
C9527	*F*	3.662	7.248	1.326	0.408	3.072	nc	nc	nc	nc
	P	0.054	**0.005**	0.326	0.799	0.068	nc	nc	nc	nc

Table 3.4: Repeated measures ANOVA for the $\Delta F/F_m'$ data of *Z. muelleri* exposed to the different concentrations of Tapis crude oil alone and Tapis crude oil + Corexit 9527 (C9527) in Corio Bay, Victoria, in summer. Degrees of freedom for interaction were exposure = 20.8, recovery = 22; for time effect exposure = 6, recovery = 7; and for concentration effect exposure = 4, recovery = 4.

Treatment	*Exposure*		*Recovery*	
	F	*P*	*F*	*P*
a. Tapis				
Concentration	4.285	**0.028**	2.991	0.073
Time	7.579	**0.011**	16.756	**0.001**
Concentration x Time	2.017	0.062	3.875	**0.003**
b. Tapis + C9527				
Concentration	4.423	**0.026**	0.882	0.509
Time	21.467	**0.000**	15.297	**0.001**
Concentration x Time	1.016	0.176	1.801	0.113

Table 3.5: One way analysis of variance (ANOVA) of $\Delta F/ F'_m$ of *Z. muelleri* exposed to the Tapis crude oil alone and Tapis crude oil + Corexit 9527 (C9527) treatments in Corio Bay, Victoria, in summer at each sampling time. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text.

Treatment		Exposure			Recovery					
		2	4	6	8	10	24	48	72	96
Tapis	*F*	1.303	1.787	2.101	6.201	4.756	6.142	3.351	1.308	4.476
	P	0.333	0.208	0.156	**0.009**	**0.021**	**0.009**	0.055	0.331	**0.025**
Tapis + C9527	*F*	1.029	2.308	1.175	6.303	6.894	1.740	0.370	0.946	3.225
	P	0.438	0.129	0.378	**0.008**	**0.006**	0.217	0.825	0.477	0.061

Table 3.6: One way analysis of variance (ANOVA) of chlorophyll *a* pigments in *Z. capricorni* exposed to Tapis crude oil, Tapis crude oil + C9527 and C9527 alone in Botany Bay, New South Wales. Values in bold denote significant difference ($P < 0.05$); values with the same numbers are similar. Averages ± SE of the mean are shown (n =3).

		Treatment	Internal control	External control	0.05 %	0.10 %	0.20 %	0.40 %	*F*	*P*
Summer	10 h	Tapis	11.1±0.6	10.6±1.6	11.2±1.6	12.2±0.8	10.3±2.0	9.0±0.2	0.652	0.872
		Tapis + C9527	13.9±2.8	12.8±1.6	13.2±2.6	14.8±3.2	11.4±1.6	18.2±1.2	1.221	0.358
		C9527	11.6±0.8	11.9±0.2	11.8±0.1	12.3±0.4	11.3±0.7	11.0±1.0	1.641	0.223
	96 h	Tapis	9.6±1.2	10±1.6	8.8±0.6	10±1.0	9.4±0.4	8.4±1.4	0.350	0.910
		Tapis + C9527	12±2.6	7.4±2.4	7.4±1.6	6.4±0.4	9.0±2.0	11.2±2.4	0.710	0.627
		C9527	**11.7±0.4[a]**	**12.5±0.7[a]**	**12.6±0.3[a]**	**12.2±0.4[a]**	**9.9±0.5[b]**	**9.8±0.2[b]**	**6.124**	**0.005**
Winter	10 h	Tapis	11.6±0.6	12.7±0.4	11.6±2.8	12.26±1.1	11.4±2.5	10.0±0.9	0.299	0.904
		Tapis + C9527	11.5±2.1	10.4±0.6	9.9±2.0	9.9±2.7	11.2±2.0	12.9±1.5	1.016	0.450
		C9527	10.3±1.6	11.3±0.4	11.3±0.5	12.3±0.0	11.1±0.5	9.4±0.7	1.548	0.247
	96 h	Tapis	11.7±1.3	11.1±1.0	8.8±1.1	11.5±1.3	10.6±1.3	10.0±1.3	0.757	0.904
		Tapis + C9527	12.1±0.7	13.2±1.1	11.0±2.6	13.4±1.4	16.5±1.5	15.1±1.3	1.673	0.215
		C9527	10.7±1.4	11.9±0.6	12.3±0.2	12±0.2	10.9±0.9	10.7±0.6	0.940	0.490

Table 3.7: One way analysis of variance (ANOVA) of chlorophyll *a* pigments in *Z. muelleri* exposed to Tapis crude oil and Tapis crude oil + C9527 in Corio Bay, Victoria. Values in bold denote significant difference ($P < 0.05$); values with the same numbers are similar. Averages ± SE of the mean are shown (n =3).

	Treatment	Internal control	External Control	0.05 %	0.10 %	0.20 %	0.40 %	*F*	*P*
10 h	Tapis	9.4±1.1	8.1±2.4	9.4±2.0	11.3±1.1	10.7±1.0	10.5±1.9	1.380	0.299
	Tapis + C9527	6.3±1.1	7.4±1.1	4.8±0.2	8.1±1.9	6.0±1.3	8.7±1.1	1.402	0.292
96 h	Tapis	**12.1±1.1^{ab}**	**11.9±1.5^{ab}**	**14.8±1.7^{a}**	**13.1±2.2^{ab}**	**10.0±1.0^{b}**	**10.8±1.6^{ab}**	**3.488**	**0.035**
	Tapis + C9527	10.2±1.3	9.1±0.9	6.7±0.7	7.9±1.2	9.9±1.1	8.1±0.3	2.606	0.081

3.3.2 IFO-380: non-dispersed, dispersed and dispersant alone

Chemical analysis

The percentage total petroleum hydrocarbon (TPH) concentrations remaining at the conclusion of the exposure period are shown in Table 3.8. All concentrations of TPH decreased significantly over the exposure day. All non-dispersed IFO-380 treatments decreased to below detection limits following the ten hour exposure period. Only the largest WAF, 0.20 and 0.40 %, concentrations of the Slickgone dispersed IFO-380 treatments had detectable amounts of hydrocarbons remaining at the end of the exposure period (Table 3.8). The highest TPH concentrations were recovered in the winter experiment, 45 % (for the 0.40 % WAF) and 30 % (for the 0.20 % WAF), with both being significantly different from the pre-exposure TPH measurement ($P < 0.01$). The TPH recovered in the summer experiments was 24 % for the 0.20 and 0.40 % WAF treatments, significantly lower than the pre-exposure concentrations ($P< 0.001$).

Table 3.8: Percent TPH remaining following 10 hour field exposure of the water accommodated fraction (WAF) of IFO-380 alone, and IFO-380 dispersed with Corexit 9527 in summer and winter in Botany Bay, New South Wales.

Treatment		WAF concentration (% remaining post-exposure)			
		0.05 %	0.10 %	0.20 %	0.40 %
IFO-380	Summer	nd	nd	nd	nd
	Winter	nd	nd	nd	nd
IFO-380	Summer	nd	nd	24 %	24 %
+ Slickgone	Winter	nd	nd	30 %	45 %

Chlorophyll *a* fluorescence

The IFO-380 alone WAF treatment in summer (Figure 3.11) did not cause any significant differences to the $\Delta F/ F_m'$ of the seagrass compared to that of the control

(Table 3.9). There was a significant effect of time ($P < 0.01$; Table 3.9) with the 0.40 % WAF significantly lower in $\Delta F/ F_m'$ than the 0.05 % WAF at four hours ($P < 0.039$; Table 3.10), but not significantly different during the last two hours of the exposure period (eight and ten hours). During the recovery days, there was a significant effect of time with the 48 hour measurement having an overall lower $\Delta F/ F_m'$ than the 72 hour measurement. The IFO-380 alone WAF treatment in winter (Figure 3.12) led to similar impacts as in the summer experiment. During the exposure day, the 0.40 % WAF was significantly lower than the control, 0.10 and 0.20 % WAF at two hours exposure, and still different to the control at four hours (Table 3.10). Over the recovery days, there was a significant effect of time ($P < 0.01$; Table 3.9). At 48 hours, the 0.40 % WAF was significantly lower in $\Delta F/ F_m'$ than the control (Table 3.10), but at no other time were concentrations significantly different to each other, suggesting minimal impact to the seagrass.

Seagrass exposed to the IFO-380 dispersed with Slickgone in summer (Figure 3.13) did not show any significant differences from the control, but some WAF concentrations were significantly different to each other ($P< 0.019$; Table 3.9). At two hour and four hours exposure the 0.40 and 0.20 % WAF both showed significantly decreased $\Delta F/ F_m'$ compared with the slightly elevated $\Delta F/ F_m'$ in the 0.05 % WAF ($P < 0.05$; Table 3.10). There was no impact to the seagrass after this and throughout the remainder of the exposure or any of the recovery days (Figure 3.13; Table 3.9). In the IFO-380 dispersed with Slickgone treatment in winter (Figure 3.14), the highest WAF concentration caused a significantly decreased $\Delta F/ F_m'$ compared to that of the control during the exposure period ($P < 0.01$; Table 3.9). The $\Delta F/ F_m'$ of the 0.40 % WAF was significantly lower than the control ($P < 0.01$) and the 0.10 % ($P < 0.05$) at two and four hours only (Table 3.10). The time effect ($P < 0.05$; Table 3.9) was a result of the four hour measurements being lower in $\Delta F/ F_m'$ than the measurements taken at eight hours exposure. During the recovery period there was minimal impact on the seagrass (Figure 3.14) with only a minor significant concentration effect ($P = 0.049$; Table 3.9) at 72 hours, the 0.05 % was significantly elevated above the 0.20 % and 0.40 % WAF concentrations. At no times were any treatments different to the control.

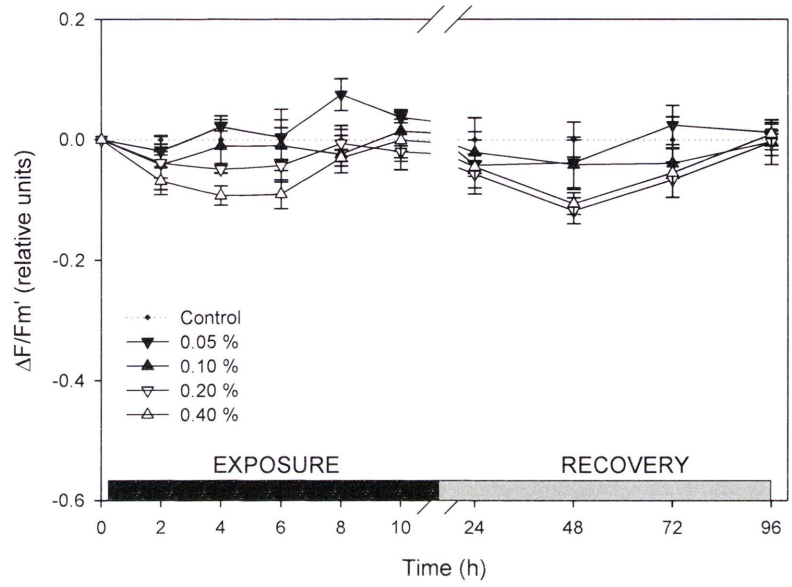

Figure 3.11: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of IFO-380 over the exposure and recovery days in summer. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

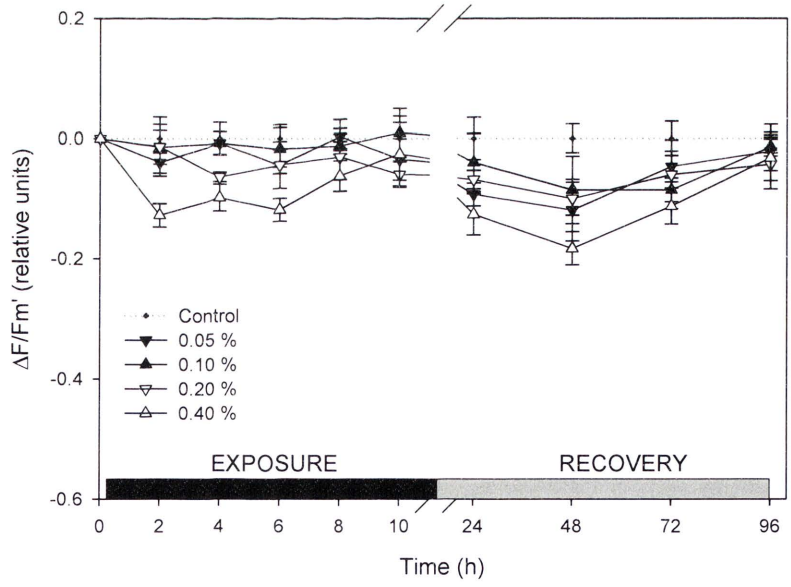

Figure 3.12: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of IFO-380 over the exposure and recovery days in winter. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

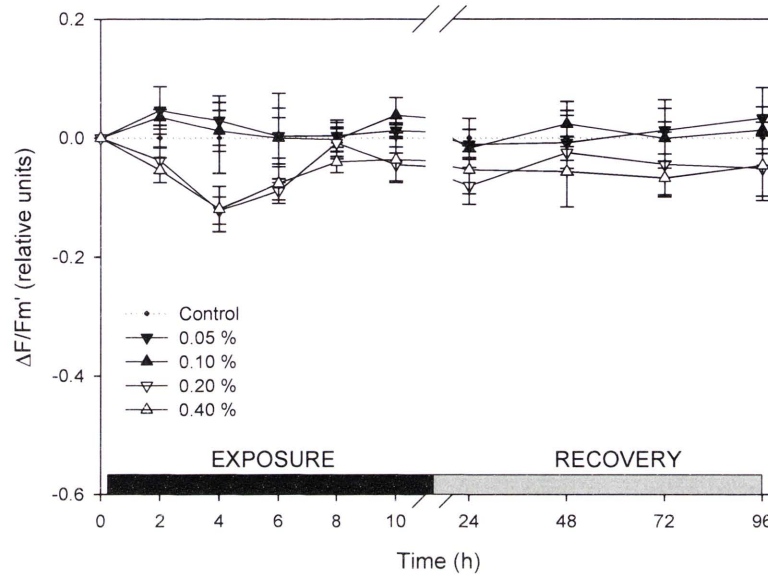

Figure 3.13: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of IFO-380 and Slickgone over the exposure and recovery days in summer. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

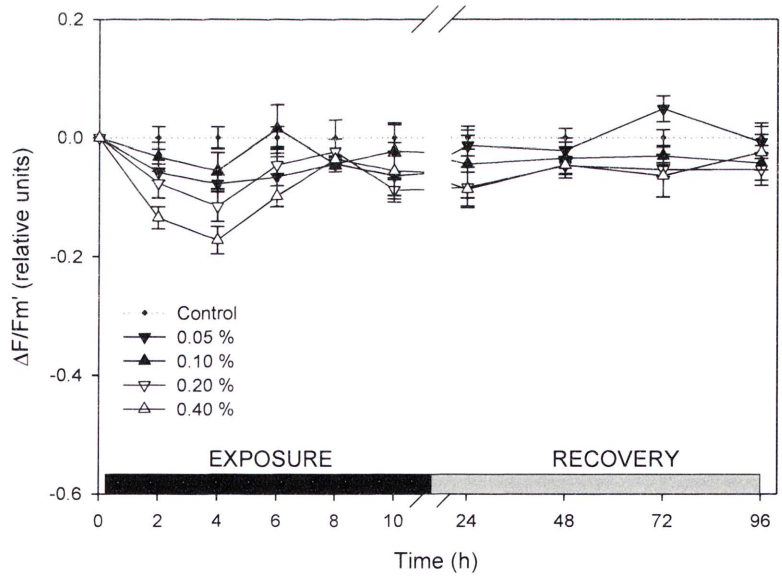

Figure 3.14: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of IFO-380 and Slickgone over the exposure and recovery days in winter. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

The Slickgone alone treatment in summer resulted in no detectable impact to the seagrass over the exposure day (Figure 3.15; Table 3.9). Over the recovery period there was a significant effect of time ($P < 0.01$; Table 3.9) with the measurements at 48 and 96 hours post treatment differing to each other. At no time did any of the WAF concentrations significantly differ from the control or to each other. The seagrass exposed to the Slickgone treatment in winter displayed minimal negative impact (Figure 3.16). No treatment differed more than 0.1 units from the control at any time during the exposure or recovery. However, there was a significant interaction effect during the exposure day (Table 3.9). In general, the higher WAF concentrations (0.20 and 0.40 %) were less than the control up to six hours, but after eight hours were slightly elevated above the control. Conversely, the 0.05 % was elevated above the control up to six hours post exposure and then decreased slightly below the control after this time. During the recovery period there was a significant time effect ($P < 0.05$; Table 3.9)with the 24 hour measurement being less than the 48 hours when the concentrations were pooled. At no time however, were the WAF concentrations significantly different from each other or from the control.

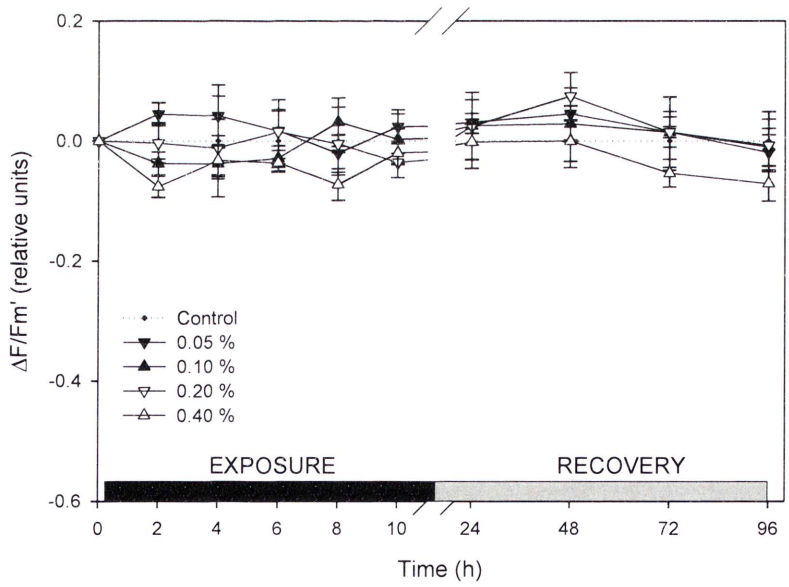

Figure 3.15: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of Slickgone over the exposure and recovery days in summer. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

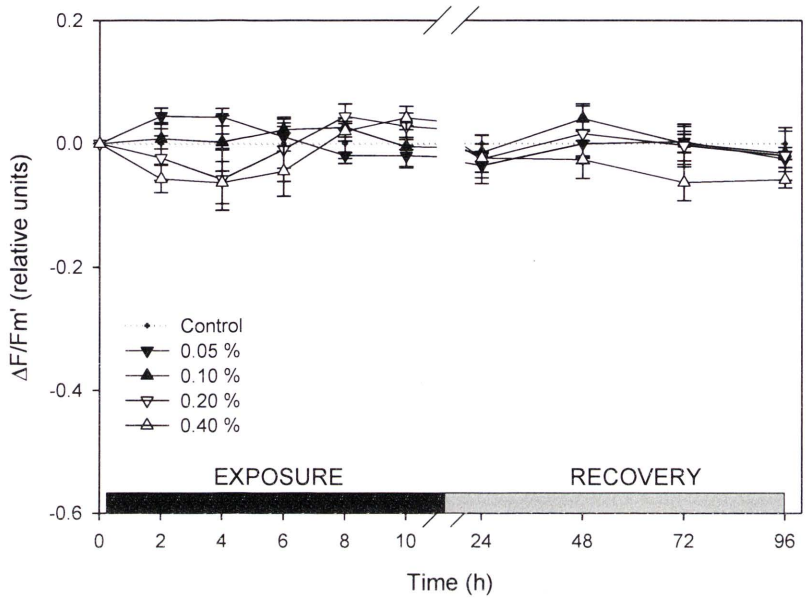

Figure 3.16: Change in effective quantum yield of *Z. capricorni* exposed to the water accommodated fraction (WAF) of Slickgone over the exposure and recovery days in winter. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (*n* =3).

Photosynthetic pigment analysis

The chlorophyll *a* photosynthetic pigment values are displayed in Table 3.11. There were no differences detected in the IFO-380, dispersed and non-dispersed, nor in the Slickgone alone WAF treatments in summer or winter Table 3.11.

Table 3.9: Repeated measures ANOVA of the effective quantum yield data of *Z. capricorni* exposed to the IFO-380 alone, IFO-380 + Slickgone LTSW and Slickgone LTSW alone treatments in summer and winter. Degrees of freedom for interaction were exposure = 20.8, recovery = 22; for concentration effect exposure = 4, recovery = 4; and for time effect exposure = 6, recovery = 7. Bold denotes significant difference at P = 0.05.

Treatment	Exposure		Recovery	
	F	*P*	*F*	*P*
Summer				
a. IFO-380				
Concentration	2.599	0.101	1.151	0.388
Time	9.959	**0.005**	7.579	**0.010**
Concentration x Time	1.100	0.410	1.177	0.357
b. IFO-380 + Slickgone				
Concentration	4.882	**0.019**	0.821	0.540
Time	2.594	0.128	1.117	0.398
Concentration x Time	1.319	0.269	0.335	0.973
c. Slickgone				
Concentration	0.679	0.622	0.480	0.750
Time	0.540	0.712	9.051	**0.006**
Concentration x Time	1.913	0.079	0.698	0.737
Winter				
a. IFO-380				
Concentration	3.697	**0.043**	2.340	0.106
Time	1.175	0.399	11.873	**0.003**
Concentration x Time	0.954	0.530	1.328	0.194
b. IFO-380 + Slickgone				
Concentration	6.309	**0.008**	3.493	**0.049**
Time	4.926	**0.033**	0.700	0.341
Concentration x Time	1.365	0.245	0.530	0.782
c. Slickgone				
Concentration	0.397	0.807	0.969	0.466
Time	8.136	**0.009**	5.063	**0.030**
Concentration x Time	3.671	**0.003**	1.517	0.193

Table 3.10: One way analysis of variance (ANOVA) of $\Delta F/ F'_m$ of *Z. capricorni* exposed to the IFO-380 alone, IFO-380 + Slickgone and Slickgone alone treatments in summer and winter at each sampling time. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text. nc denotes ANOVA not calculated (no significant difference in the rmANOVA - Table 3.5).

Treatment		Exposure					Recovery			
		2	4	6	8	10	24	48	72	96
Summer										
IFO-380	*F*	1.825	3.628	1.947	2.512	0.638	0.473	2.457	1.268	0.135
	P	0.201	**0.045**	0.179	0.108	0.647	0.755	0.114	0.345	0.966
IFO-380 +	*F*	6.065	5.227	2.712	0.572	2.521	Nc	nc	nc	nc
Slickgone	*P*	**0.010**	0.960	0.091	0.689	0.107	Nc	nc	nc	nc
Slickgone	*F*	nc	Nc	nc	Nc	nc	0.156	0.657	0.642	0.599
	P	nc	Nc	nc	Nc	nc	0.956	0.636	0.645	0.672
Winter										
IFO-380	*F*	5.081	4.927	2.769	1.710	0.621	2.183	2.751	2.267	0.526
	P	**0.017**	**0.019**	0.087	0.224	0.658	0.144	0.089	0.134	0.720
IFO-380 +	*F*	6.095	7.929	3.552	0.878	0.920	1.355	0.958	6.402	0.679
Slickgone	*P*	**0.009**	**0.004**	**0.047**	0.511	0.490	0.316	0.471	**0.008**	0.622
Slickgone	*F*	2.114	1.657	0.530	1.524	1.348	0.640	1.537	1.295	0.507
	P	0.154	0.235	0.717	0.268	0.319	0.646	0.264	0.336	0.732

Table 3.11: One way analysis of variance (ANOVA) of chlorophyll *a* pigments in *Z. capricorni* exposed to IFO-380, IFO-380 + Slickgone and Slickgone alone at ten hours and 96 hours in summer and winter. Values in bold denote significant difference at P = 0.05; values with the same numbers are similar. Averages ± SE of the mean are shown (*n* =3).

		Treatment	Internal control	External control	0.05%	0.10%	0.20%	0.40%	*F*	*P*
Summer	10 h	IFO-380	14.0 ± 1.4	13.6 ± 1.4	12.6 ± 0.8	12.7 ± 0.9	13.6 ± 1.5	11.2 ± 0.3	0.795	0.574
		IFO-380 + Slickgone	12.9 ± 1.2	12.0 ± 1.5	12.7 ± 0.3	13.3 ± 0.2	13.1 ± 0.9	11.1 ± 1.1	1.354	0.308
		Slickgone	12.6 ± 1.1	11.7 ± 0.5	12.0 ± 1.0	13.3 ± 0.3	12.7 ± 1.0	11.3 ± 0.7	0.750	0.602
	96 h	IFO-380	13.3 ± 1.5	12.2 ± 0.6	11.9 ± 0.3	11.4 ± 0.1	11.5 ± 0.8	11.5 ± 0.6	1.685	0.213
		IFO-380 + Slickgone	11.0 ± 0.2	13.2 ± 0.8	14.0 ± 0.5	13.2 ± 0.5	12.3 ± 1.3	11.3 ± 0.7	1.431	0.282
		Slickgone	11.0 ± 0.2	13.9± 0.4	12.8 ± 1.6	13.1 ± 0.6	11.8 ± 1.4	11.3 ± 0.3	1.481	0.267
Winter	10 h	IFO-380	12.1 ± 2.7	11.0 ± 0.8	11.1 ± 0.2	10.8 ± 0.8	11.9 ± 0.7	11.6 ± 0.5	0.903	0.510
		IFO-380 + Slickgone	14.3 ± 1.7	10.7 ± 0.5	13.2 ± 0.9	13.7 ± 1.0	14.2 ± 1.6	10.1 ± 2.7	0.690	0.641
		Slickgone	12.6 ± 1.3	12.1 ± 0.3	12.2 ± 0.5	12.3 ± 0.5	11.6 ± 0.6	11.2 ± 0.4	0.653	0.665
	96 h	IFO-380	12.5 ± 0.3	11.3 ± 0.6	12.1 ± 0.8	11.1 ± 0.5	10.7 ± 0.6	10.4 ± 0.7	0.174	0.967
		IFO-380 + Slickgone	11.3 ± 0.9	14.3 ± 1.7	14.7 ± 0.4	13.9 ± 0.5	13.9 ± 1.9	10.8 ± 2.0	2.531	0.087
		Slickgone	12.2 ± 2.1	12.8 ± 0.6	12.4 ± 0.5	11.7 ± 0.7	10.7 ± 0.6	10.0 ± 0.7	1.137	0.393

3.4 Discussion

The addition of dispersants to oil slicks, by design, increases the petroleum hydrocarbon concentration in the water column (Yamada *et al.* 2003). The results of this study show that this does little by way of increasing the toxicological effect to subtidal seagrass. Neither the oil, dispersed oil or dispersant alone treatments resulted in any great impact to the seagrass in summer or winter. The magnitude of decrease in $\Delta F/ F_m'$ of *Z. capricorni* in the dispersed oil treatments (crude and IFO-380) was greater than the decrease in the oil alone or dispersant alone treatments, but this was only marginally so (within 0.1 units $\Delta F/ F_m'$). In some petrochemical treatments the $\Delta F/ F_m'$ of the seagrass was actually enhanced. The results showed that where negative responses were detected in the different petrochemical treatments, they were only in the highest WAF concentrations, mostly in the 0.40 % WAF concentration, and in some experiments also in the 0.20 % WAF concentration. Most importantly, the seagrass showed complete recovery following any negative impact.

No treatment led to an impact of greater than 0.2 units $\Delta F/ F_m'$ from the control. The initial (pre-exposure) $\Delta F/ F_m'$ was greater than 0.6 units in all samples. This means that no treatment exhibited a response greater than 30 % inhibition, with most showing a maximum response of far less than 20 % (0.1 units below the control). In a field study by Macinnis and Ralph (2003b), the $\Delta F/ F_m'$ of *Z. capricorni* decreased more than 0.5 units following two hours exposure to a 0.25 % water accommodated fraction (WAF) of Champion crude oil. The decrease in the Macinnis and Ralph (2003b) study was far larger and from a lower concentration of WAF than in this study. Haynes *et al.* (2000) found the $\Delta F/ F_m'$ of *Z. capricorni* to decrease to a maximum of about 0.4 units over five days exposure to 100 $\mu g\ L^{-1}$ diuron (Haynes *et al.* 2000). It is evident from previous research that pollutants, including petrochemicals, can decrease the $\Delta F/ F_m'$ of *Z. capricorni* quite dramatically, so it seems reasonable to suggest that the petrochemicals used in this study were simply not very, or not at all, toxic to the seagrass.

There was some evidence of seasonal variation between the summer and winter results for *Z. capricorni* but again, a lack of any great decrease in $\Delta F/ F_m'$, meant this was only minor. It must be remembered that there was no seasonal replication of treatments. Due to the logistical intensity of conducting the experiments, each treatment was conducted only once in summer and once in winter, so no statistical comparisons were made. However, the magnitude of decrease in $\Delta F/ F_m'$ in the winter experiments was commonly greater than that observed in the summer experiments. This was true for the crude alone, IFO-380 alone, IFO-380 dispersed with Slickgone, and the Corexit 9527 alone treatments. Again, these differences were generally within 0.1 units $\Delta F/ F_m'$ of their summer counterparts. The crude and IFO-380 alone treatments had no detectable levels of hydrocarbons remaining at the end of the exposure period in both seasons, providing no evidence to support any seasonal differences in petrochemical breakdown. However, the crude dispersed with Corexit 9527 and the IFO-380 dispersed with Slickgone did show slightly greater recovered amounts in winter in support of studies suggesting that petrochemical breakdown can be influenced by such factors as temperature variations (Burridge & Shir 1988; Nemr 2000; Singer *et al.* 2000). The chlorophyll *a* pigment concentrations showed no evidence of any seasonal variability in the effects to the seagrass. Although, significant differences in the chlorophyll *a* pigment concentrations of *Z. capricorni* were only detected in the Corexit 9527 alone treatment in summer where no difference in the $\Delta F/ F_m'$ data occurred. It remains unclear as to why the chlorophyll pigment concentrations differed in the Corexit 9527 treatment, although the general findings regarding this method are similar to those described by Macinnis and Ralph (2003) in that it may simply be less sensitive than chlorophyll *a* fluorescence techniques in detecting seagrass stress from petrochemicals. In summary, seasonal variation in the response of Z. *capricorni* to these petrochemicals appears slight, but likely, and supports other research findings on seasonal variation in plant sensitivity from oil pollution (eg. Pezeshki *et al.* 2000) and to seagrass (Brun *et al.* 2002).

Zostera muelleri was not negatively impacted by the petrochemical treatments. The seagrass actually displayed a significant increase in $\Delta F/ F_m'$ in most of the concentrations for both non-dispersed and dispersed crude oil. The chlorophyll *a*

pigment concentration of the seagrass exposed to the crude alone treatment also increased, but not until 96 hours following the initial exposure. The $\Delta F/ F_m'$ in the chamber treatments was greater than the $\Delta F/ F_m'$ of the chamber control; and both chamber treatments and chamber controls, were greater than the external control. This implies two things. Firstly, there was an interaction between the seagrass and the petrochemicals within the chambers; and secondly, the chambers did have an impact on the photosynthetic output of the seagrass. There are several suggested reasons for this outcome in Corio Bay, mainly arising from the depth of the chambers.
Port Phillip Bay has a maximum tidal range of about one metre (Jenkins & Wheatley 1998) and whilst conducting these experiments, the depth of the chambers ranged from just below, to about 50 cm below, the water surface. The temperature within the chambers was measured intermittently during the exposure day of both treatments and was found to be 2-3 °C greater than the surrounding water just after noon (data not shown). Temperature was not measured in the Botany Bay experiments as the chambers were greater than 2 m below the water surface at times, due to the greater tidal range within the bay. The maximum light intensities and temperatures to which *Z. muelleri* was subjected to in Corio Bay, were likely to have been far greater than those experienced by *Z. capricorni* in the deeper waters of the Botany Bay experiments.

Seagrass growth has been shown to increase with increasing water temperature up to the maximal thermal limit of the seagrass (Masini *et al.* 1997) and it is likely that this played some role in the enhancement of $\Delta F/ F_m'$ observed in the chambers in the Corio Bay experiments. The increased temperature may have increased the rate of microbial breakdown of the oil (Nemr 2006) however the increased light intensities would also have had the potential to increase the photooxidation of the PAHs (Garrett *et al.* 1998; Kirby *et al.* 2007). Microbial breakdown of the oil may also have been enhanced due to high levels of nutrient runoff within Corio Bay (Zann 1994). Oil concentration may have been reduced leading to the temperature in the chambers dictating the enhancement of the $\Delta F/ F_m'$ of the seagrass. However, regardless of the mechanisms of petrochemical breakdown, the fact that the $\Delta F/ F_m'$ of the seagrass in the WAF treatments was greater than that observed in the control chamber controls suggests that

there is some link between the temperature of the water column and the toxic potential or affect of the petrochemical to the seagrass.

The addition of dispersant to an oil spill is known to increase the bioavailability of the petrochemical, but the counter concern is often the potential detrimental effect of the greatly increased TPH in the water column. There was clearly an increase in TPH in the WAF with the addition of dispersants in this study (Chapter Two, Figures 2.2 & 2.4), and there were measurable amounts of hydrocarbons in the dispersed oil treatments at the end of the exposure period; yet there was no great difference in photosynthetic health ($\Delta F/ F_m'$, chlorophyll *a* pigment analysis) to *Z. capricorni* or *Z. muelleri*. Baca and Getter (1984) found that dispersant application increased the hydrocarbon concentration in the water column more than 50 fold, yet found *Thalassia testudinum* was far greater impacted by the non-dispersed oil. The minor effects to either species in this study from either non-dispersed or dispersed oil suggests a rapid breakdown of oil in the water column or sediments (Dodd 1974; Leahy & Colwell 1990; George-Ares & Clark 1995) or that the seagrasses were quite resilient to these petrochemicals.

In most treatments, in both localities, there were none, or only minimal levels of hydrocarbons recovered following the exposure period, and this result is similar to minimal or no hydrocarbons being detected after 24 hours by Hatcher and Larkum (1984). Although, the shallow nature of the Corio Bay experiments may have altered the rate of breakdown of the hydrocarbons (Nemr 2006) within the experimental chambers, the similar levels of loss that occurred within the respective Botany Bay experiments provides little substantiation of this. The incorporation of hydrocarbons into the sediment (Page *et al.* 1999; Fingas 2001) and microbial breakdown of oil (Leahy & Colwell 1990; Venosa & Holder 2007) are more likely scenarios for this significant loss over the exposure period. There was minimal potential for evaporative loss in these experiments as the mesocosms were submerged at all times. However, the amount of petrochemicals that adhered to the mesocosm itself must also be considered. In this study, there was some visual evidence of petrochemical adherence to the mesocosm lid and the internal walls in some treatments. Clark and Noles (1994) reported that partitioning of pollutants along the walls of test systems is a common occurrence in

mesocosm designs and it is likely in this study, that at least some of the 'loss' of petrochemicals over the exposure day was due to this partitioning.

Due to the limited solubilities of many hydrocarbon fractions, only a portion of the oil has the potential to enter the water column (Ali *et al.* 1995; Page *et al.* 2000; Groner *et al.* 2001). Following the *North Cape* oil spill, Reddy and Quinn (1999) detected water column concentrations of oil up to 3.9 mg L^{-1} and reported them as some of the highest ever reported. Kim *et al.* (2010) found oil concentrations as high as 16.6 mg L^{-1} 20 cm below the water surface in the first few days following the *Hebei Spirit* oil spill (2009). However, in extreme oil spill events, water column concentrations can be even higher, especially in weather-protected environs. For example, water column concentrations in estuarine regions following the *Amoco Cadiz* spill were as high as 500 mg L^{-1} (Seymour & Geyer 1992). Prior to exposure, but following weathering, the total petroleum hydrocarbon (TPH) concentration of the 1.00 % WAF treatments in this study ranged from 3 to 317 mg L^{-1}; the BTEX components ranged from less than 1 mg L^{-1} to 11 mg L^{-1}; and naphthalene ranged from less than 2 mg L^{-1} to 130 mg L^{-1} (Chapter Two). While a direct extrapolation of these values cannot be made to the concentrations used in these *in situ* experiments (highest concentration was 0.40 % WAF), it is estimated that the initial concentrations of petrochemicals in the water column were quite high, yet there was little impact to the seagrass.

It is interesting to note, that several hours following the initial severe decline in $\Delta F/ F_m'$ in the Macinnis and Ralph (2003b) study, the seagrass had actually recovered. Although on a less dramatic scale, the same result occurred within this study. Where impacts were detected in this study, generally between two and eight hour's exposure, they were always followed by recovery of the seagrass. Similarly, Hatcher and Larkum (1982) found only short term effects to *Posidonia australis* following petrochemical exposure with oxygen production and consumption rates returning to pre-treatment levels. Long-term studies also support a lack of evidence for long–term effects to seagrass meadows. Kenworthy *et al.* (1993) found no differences to seagrass after a year following the Gulf War Spill, whilst Dean *et al.* (1998) found no impacts one year after the Exxon Valdez spill.

From this information it is suggested that the *Z. capricorni* and *Z. muelleri* may, in fact, be quite resilient to the petrochemicals used in this study, when exposed to these concentrations, under the ten hour exposure conditions. However, it is clear that the largest treatments used here, 0.40 % WAF, did show some negative impact to *Z. capricorni* and an enhanced growth response from *Z. muelleri*; and so it was deemed useful to analyse greater concentrations to determine whether these would result in an increased, or any, detrimental effect to the seagrass. The severe damage to *Thalassia* spp. following the spill of crude oil (Naduau & Berquist 1977) provided clear evidence that at a certain petrochemical concentration, under certain conditions, subtidal seagrass can be severely impacted by petrochemicals. If a spill greater than what the largest treatment used in this study represented were to occur, (eg. *Amoco Cadiz* spill) extrapolation of these results would not be very useful. Sometimes an upper benchmark of impact is useful to provide more information as to effects to organisms, even when above realistic environmental conditions.

These field experiments were labour intensive and logistically difficult to conduct. Furthermore, changing weather conditions often resulted in differing wave heights, sea surface conditions and light attenuation within the ten hour exposure period, the recovery days and between the different experiments. Laboratory experiments would enable the controlling of these variables and allow for more treatments, greater concentrations and other relevant species to be analysed to provide a more comprehensive picture of the effects of petrochemicals to seagrass. Laboratory experiments replicating these field experiments form the next chapter, Chapter Four.

4 Impacts of Petrochemicals in Laboratory Experiments

4.1 Introduction

Laboratory experiments have been shown in some circumstances to overestimate (Clark & Noles 1994; Macinnis-Ng & Ralph 2003b) whilst in others to underestimate (Connell *et al.* 1999) the toxicological impacts of contaminants to marine organisms compared with field estimates. The complex interplay of environmental parameters within the field are difficult can to replicate exactly in a laboratory experiment (Clark & Noles 1994; Hemminga & Duarte 2000). However, laboratory experiments are commonly used in ecotoxicological studies for several major reasons. Laboratory experiments provide more control in experimental conditions. Further, they are less logistically demanding than field experiments. Because of this, more test variables can be analysed including more species and more petrochemical treatments, and test organisms can be exposed to a greater range of concentrations.

Laboratory experiments allow for greater control than that which would occur in the field (Clark & Noles 1994). Laboratory experiments conducted on different days will not be affected by different environmental conditions such as changing light levels or alteration in seawater temperature. Oils are affected by the degree of sunlight exposure which can increase the toxicity of polycyclic aromatic hydrocarbons (PAH), amongst other components, to organisms (NRC 2005; Kirby *et al.* 2007). Similarly, the properties of petrochemicals can change because of changes to seawater temperature (Singer *et al.* 2000; Nemr 2006). For example, elevated seawater temperature can decrease the viscosity of oils, increasing the immediate toxicity to organisms and increasing the rate at which these toxic components are lost via evaporation and degradation (API 1999; Singer *et al.* 2000; NRC 2005). Clearly, to be able to control the environmental variation when assessing different petrochemical treatments is highly valuable in terms of improved reliability.

Another advantage of laboratory experiments is that a greater number of species can be assessed. Some species are difficult to assess in the field due to logistical issues associated with their habitat or temporal or spatial patchiness. For example, *H. ovalis* displays both temporal and spatial patchiness in Botany Bay (Larkum & West 1990; Zann 1996). Further, where a species is patchily distributed, introducing pollutants into their environment for scientific purposes may result in damage to the habitat for that particular species. Considering there is a wide diversity in response of organisms from petrochemicals, assessing more than one species is most useful, especially for developing management guidelines.

Studies have shown different responses from different species of seagrass with petrochemical exposure. For instance, Thorhaug *et al.* (1986) found *Thalassia* was more tolerant to oil and dispersed oil than *Halodule* or *Syringodium*. Haynes *et al.* (2000) also found differences between species when assessing the impacts of the herbicide, diuron. They found that *H. ovalis* was impacted at lower concentrations of diuron than either *Z. capricorni* or *Cymodocea serrulate*. One reason for response differences between species of seagrass is likely due to morphological variations (Duarte *et al.* 1994; Vermaat *et al.* 1997).

Zostera capricorni and *Halophila ovalis* are two morphologically different species that are commonly found in the same area and are both potentially at risk from oil spills. The two species vary in their leaf shapes which may act to alter the rate of uptake or adherence of oils to the seagrass. *Halophila ovalis* has been shown to be one of the most sensitive species in experiments assessing a wide range of environmental variables and pollutants such as exposure to UV irradiance (eg. Haynes *et al.* 2000; Enriquez *et al.* 2002). However, the effects from petrochemicals to *H. ovalis* have been shown to be minor in other studies (Durako *et al.* 1993; Ralph & Burchett 1998a). Furthermore, several studies suggest *Z. capricorni* is a moderately resilient species. Larkum and West (1990) found that Z. *capricorni* in Botany Bay had colonised areas previously inhabited by *Posidonia australis*. In petrochemical studies, Macinnis and Ralph (2003b) showed minimal impact to *Z. capricorni* from non-dispersed and dispersed crude oil. The

assessment of these two species under the same experimental conditions would greatly assist furthering scientific knowledge of petrochemical impacts to seagrass.
This chapter describes a range of experiments to determine the effect of petrochemicals on seagrass under laboratory conditions. The major aims of this chapter were to determine the effects of non-dispersed and dispersed oil on *Z. capricorni* and *H. ovalis* in a laboratory experiment. The specific objectives were to 1) determine the effect of petrochemical treatments at different concentrations; 2) determine the nature of response in relation to the level of impact, time of impact and recovery and 3) determine the effectiveness of a semi-quantitative measure of hydrocarbon concentration at detecting these impacts.

4.2 Methods

Whole plant laboratory experiments were designed to replicate field experiments (Chapter Three). However, to increase the utility of these experiments, additional treatments were investigated and two morphologically different species were analysed.

Experimental Design

Fifteen clear glass aquaria (210 mm (W) x 355 mm (L) x 200 mm (H)) were set up in a temperature controlled laboratory (20 ± 1 °C). The aquaria were maintained under 200 photons m^2 s^{-1} of light on a 16: 8 light: dark cycle. Each tank was aerated with two small air stones. Seagrass, *Z. capricorni* and *H. ovalis*, were held in plastic trays (70 mm x 120 mm) with about 40 mm of natural sediment topped with 10 mm of fine sand, within the tanks. Aquaria were filled with seawater (via UTS laboratory storage supply sourced from Rose Bay, Sydney Harbour) and the appropriate amount of water accommodated fraction (WAF) to create the specific petrochemical treatment concentrations required was added. To reduce evaporation and to better replicate the mesocosms in the field, 5 mm thick acrylic sheets cut to size were placed on top of the tank as a lid. Two small holes were drilled through the lid to allow the 2 mm fibre optic (Poly Optics Australia) for the chlorophyll *a* fluorescence measurements to remain in

the same place throughout the exposure period. As such each measurement performed during the exposure period was performed at the same position, on the same blade.

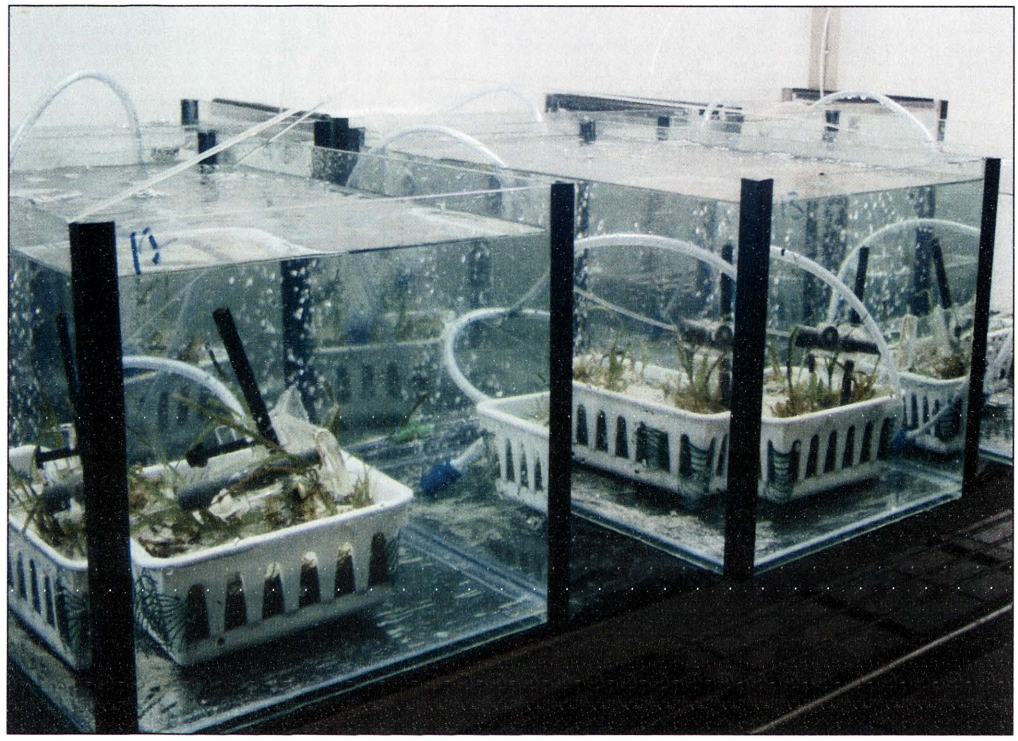

Figure 4.1: Photo of tank set-up for the whole plant laboratory exposure experiments. N.B. Tank lids have been removed.

The WAF treatments were added at 0700 hours. At 1700 hours, tanks were completely drained and washed, and then filled with fresh seawater, to simulate a recovery period. Airstones were also washed at this time. Chlorophyll *a* fluorescence measurements, effective quantum yield ($\Delta F/ F'_m$) were taken prior to exposure and then every two hours throughout the exposure period. At the end of the exposure period, a coloured thread was loosely tied around the seagrass blades to ensure measurements were performed on the same ramet of seagrass during the recovery period. Recovery period measurements were taken once daily at approximately 0700 hours for four consecutive days. As with the *in situ* experiments, Chapter Three, the recovery period was incorporated to assess seagrass response following the replenishment of 'fresh' seawater.

Water Accomodated Fraction

Concentrations of the water accomodated fraction were greater than those used in the *in situ* field experiments (Chapter Three) for two reasons. The first was that the low concentrations, 0.05 % and 0.10 % WAF, elicited minimal, if any, response in initial laboratory trials. The second reason was to elicit a strong response to use as an upper benchmark for demonstration of petrochemical impact. For these reasons the concentrations used in this section of experiments were 0.20 %, 0.40 %, 1.00 % and 2.00 % WAF.

Field experiments (Chapter Three) were constrained by weather conditions and daily tidal fluctuations which limited the amount of treatments, and the concentrations of those treatments, that could be applied. In the laboratory, however, there were fewer experimental constraints, thereby, two extra dispersed oil and dispersant alone treatments (four extra treatments) were applied. Furthermore, exposing the higher concentrations in the field was considered potentially environmentally damaging to the seagrass and surrounding biota, and was thus not approved under the permit conditions. Laboratory exposure treatments were Tapis crude oil; Tapis crude oil with Corexit 9527; Tapis crude oil with Ardrox; Corexit 9527 alone; Ardrox alone; IFO-380; IFO-380 with Slickgone LTSW; IFO-380 with Corexit 9500; Slickgone LTSW alone and Corexit 9500 alone.

Chemical Analysis

Water samples (15 ml per tank) were taken prior to exposure and at the end of the exposure period. Three samples were taken from each tank and were averaged to give a single value per tank. Samples were analysed immediately using an "oil-in-water" fluorometer with the Crude Oil Module (Turner Trilogy Laboratory Fluorometer, Turner Designs, USA). The crude oil module is set at fixed wavelengths (from 400 to 600 nm) and determination of total petroleum hydrocarbons (TPH) is based on the fluorescence within this broad wavelength range. The fluorometer is an oil-in-water

fluorometer, and as such samples were analysed without performing any extraction procedures.

The pre- and post-exposure measurements in this study were used to derive a value of percent TPH remaining at the conclusion of the exposure period. The fluorometer was first calibrated with the pre – exposure WAF treatments (0.00 %, 0.20 % 0.40 %, 1.00 % and 2.00 % WAF concentration). Comparisons of the percentage remaining values were also made between concentrations within the same treatment and between different petrochemical treatments.

Photosynthetic Pigment Analysis

The determination of chlorophyll *a* pigment concentration was performed using a fluorometer (Chlorophyll *a* non-acidification module, Turner Trilogy Laboratory Fluorometer, Turner Designs, USA). Samples were collected at 10 and 96 hours and were stored and extracted using the same methods as described in Chapter Three (Section 3.2.4). The fluorometer was calibrated with chlorophyll *a* standards (Turner Designs, USA).

4.3 Results

4.3.1 Tapis crude oil: non-dispersed, dispersed and dispersant alone

4.3.2 *Total petroleum hydrocarbon (TPH) concentration*

Figure 4.2 displays the percentage total petroleum hydrocarbon (TPH) concentration remaining following ten hours exposure under the laboratory conditions. Results were analysed to test if there was a significant difference in the TPH between pre-exposure levels and following the ten hour exposure period. Table 4.1 displays the statistical analysis (*t* tests) of the pre- and post-exposure values of the water soluble fraction for each treatment. All 0.00 % concentrations (controls) showed some, although very low,

fluorescence. The controls are not included in the figures but statistical analysis is provided inTable 4.1.

There was minimal loss of total petroleum hydrocarbons (TPH) in the crude alone WAF treatments over the exposure period (Figure 4.2). Only the 0.20 % and 1.00 % concentrations showed a significant difference between the pre- and post-exposure measurements with the 1.00 % concentration having lost 23 % of the TPH by the end of the exposure period (Table 4.1). The two dispersed crude oil treatments displayed very different results from each other. The Corexit 9527 dispersed crude (Figure 4.2) showed no significant difference between the pre- and post-exposure TPH measurements (Table 4.1). The Crude dispersed with Ardrox (Figure 4.2) showed highly significant reductions in every concentration (Table 4.1). The TPH in the 0.40 %, 1.00 % and 2.00 % concentrations in the crude dispersed with Ardrox decreased to almost half of the initial TPH concentration.

The two dispersant alone treatments, Corexit 9527 and Ardrox alone (Figure 4.2), produced similar results to each other with both treatments showing minimal loss over the exposure period. Both treatments displayed significant increases in the 1.00 % concentration over the exposure period (Table 4.1). The 2.00 % Ardrox treatment decreased significantly, but this was only minor, approximately 10 % less than the initial exposure amount.

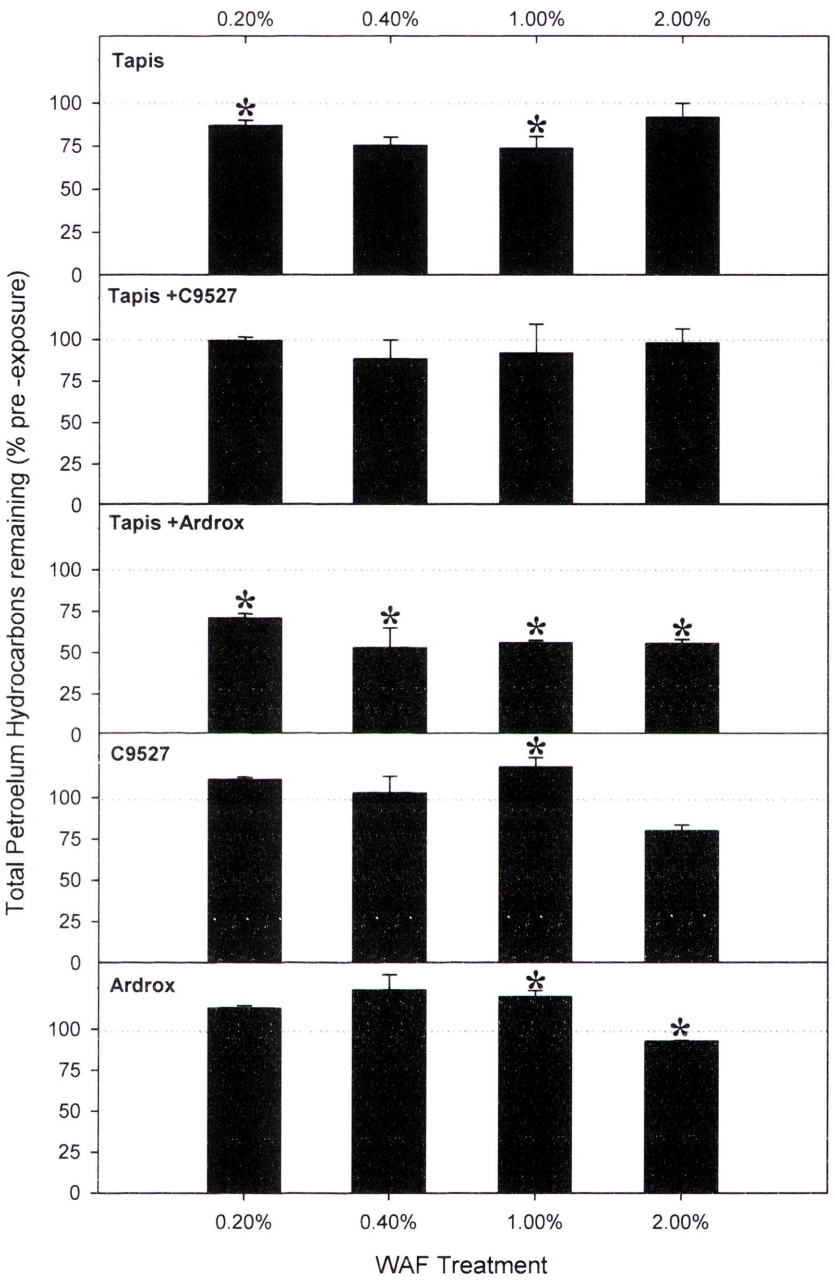

Figure 4.2 Percentage TPH remaining after ten hours exposure determined by Ultra-violet fluorescence for the Tapis crude oil, Tapis crude oil + C9527; Tapis crude oil + Ardrox; C9527 and Ardrox WAF treatments (* denotes a significant difference between the pre- and post concentrations. Average ± standard error of the mean are shown (n = 3).

Table 4.1 Independent *t* test analysis of the total hydrocarbon concentration following ten hours exposure of Tapis crude oil alone; Tapis crude oil + Corexit 9527 (C9527); Tapis crude oil + Ardrox; C9527 alone and Ardrox alone WAF treatments. Values in bold denote significant differences at P = 0.05.

Treatment	Concentration							
	0.20 %		0.40 %		1.00 %		2.00 %	
	T	*p*	*t*	*p*	*t*	*p*	*t*	*P*
Crude	2.60	0.12	*2.94*	*0.10*	*2.94*	*0.10*	0.77	0.52
Crude + C9527	0.05	0.96	0.60	0.61	0.28	0.81	0.11	0.92
Crude + Ardrox	3.68	0.06	3.24	0.08	46.38	**0.00**	11.18	**0.01**
C9527	1.85	0.14	0.13	0.91	20.18	**0.00**	3.03	0.09
Ardrox	6.59	**0.02**	2.56	0.12	5.97	**0.03**	6.59	**0.02**

4.3.2.1 Zostera capricorni

Chlorophyll a *fluorescence*

The crude alone WAF treatment elicited no detectable response to the $\Delta F/F_m'$ of *Zostera capricorni* over the exposure or recovery days (Figure 4.3; Table 4.2). The $\Delta F/F_m'$ in the 1.00 % WAF concentration was depressed below the other concentrations especially at 24 hours, but this was not significantly different to any other concentration.

The crude dispersed with Corexit 9527 WAF treatment elicited a minimal response from *Z. capricorni* with some photosynthetic impact detected towards the end of the exposure day (Figure 4.4;Table 4.2). There was a highly significant time effect ($P < 0.01$) due to differences between the four hour and eight hour measurements and a significant concentration effect ($P< 0.05$). One way ANOVAs (Table 4.3) determined differences between concentrations at six ($P = 0.048$) and eight hours exposure ($P = 0.002$) with the 2.00 % significantly lower than the control. There were no further impacts to the seagrass at ten hours nor in any of the recovery days (Table 4.2).

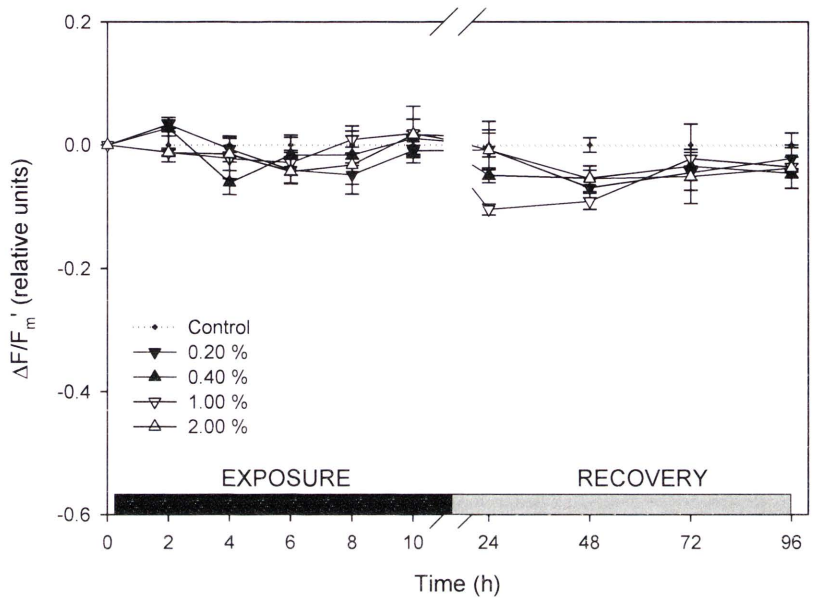

Figure 4.3: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of Tapis crude oil. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

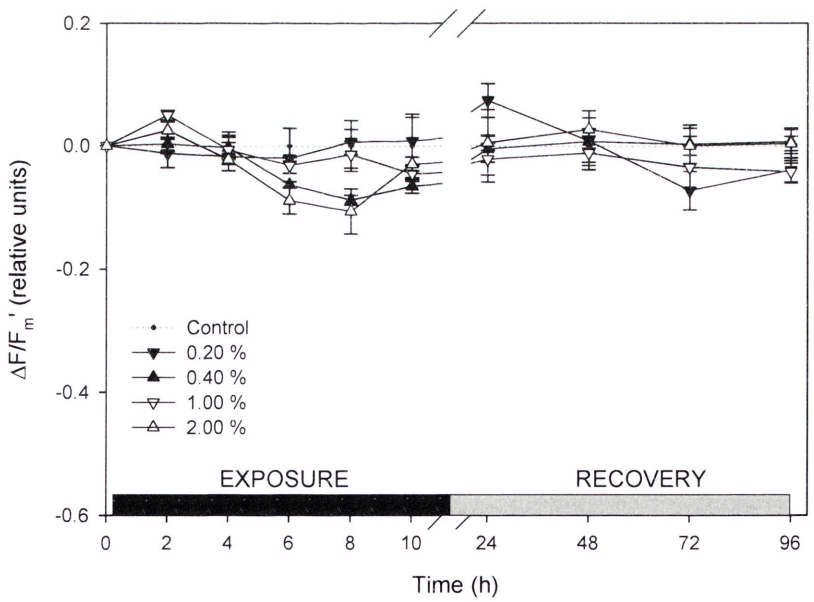

Figure 4.4: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of Tapis crude oil and Corexit 9527. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

Z. capricorni was impacted negatively by the crude dispersed with Ardrox treatment. This negative impact developed towards the end of the exposure day and during the recovery days (Figure 4.5; Table 4.2). At six hours the 0.20 % became significantly elevated above the control ($P < 0.029$; Table 4.3). At eight and ten hours exposure, the $\Delta F/F_m$' exposed to the 2.00 % WAF concentration was significantly depressed from all other treatments. Over the recovery days the $\Delta F/F_m$' of the 2.00 % WAF concentration was significantly lower ($P < 0.01$) than all other concentrations including the control at 24, 48 and 72 hours, but not at 96 hours. At 48 and 72 hours the overall $\Delta F/F_m$' of the seagrass was depressed when compared with the first recovery day, 24 hours ($P = 0.037$; Table 4.2).

In the Corexit 9527 alone treatment (Figure 4.6), the $\Delta F/F_m$' of *Z. capricorni* exposed to the 2.00 % concentration was significantly different to the control overall at two and six hours ($P < 0.01$; Table 4.2; Table 4.3). One way ANOVAs detected the $\Delta F/F_m$' of the seagrass exposed to the 2.00 % concentration was significantly different to the control at two and six hours, whilst at eight hours the $\Delta F/F_m$' of the seagrass exposed to the 0.40 % concentration was also significantly different to the control. There were fluctuations in the seagrass response from the lower concentrations up to six hours and a somewhat decreasing response during the last four hours. The seagrass response at the two hour measurements was significantly different to the last experimental measurement with this decrease in $\Delta F/F_m$'. All concentrations were significantly different to the control at 24, 48 and 72 hours with the 2.00 % still significantly different at 96 hours. The first two days during the recovery were significantly different to the last two days of the experiment. Over the last two days of measurements the low concentrations began to increase in $\Delta F/F_m$'.

In the Ardrox alone treatments (Figure 4.7; Table 4.2) there were significant interaction effects during the exposure day and recovery periods. There were fluctuations in the $\Delta F/F_m$' measurements at different times throughout the experiment ($P < 0.01$; Table 4.2). Significant differences in the $\Delta F/F_m$' of the seagrass between concentrations began at four hours exposure with the 0.40 %, 1.00 % and 2.00 % concentrations significantly different to the control at this time (Table 4.3). The $\Delta F/F_m$' in the seagrass exposed to

the 1.00 % concentration remained significantly different to the control throughout the remainder of the exposure day, whilst the $\Delta F/F_m$' in the 2.00 % concentration was significantly different to the control at 10 hours. Over the recovery period, the $\Delta F/F_m$' in the 0.20 % was elevated and the $\Delta F/F_m$' in the higher concentrations were significantly lower than this treatment and the control over most of the recovery period. The $\Delta F/F_m$' in the 0.40 %, 1.00 % and 2.00 % treatments was significantly less than the control at 96 hours ($P \leq 0.01$).

Photosynthetic Pigment Analysis

The results of the chlorophyll *a* pigment analyses of *Z. capricorni* conducted at 10 and 96 hours following exposure to the crude alone, dispersed crude and the dispersant alone treatments are presented in Table 4.4. There were no significant differences detected in this species at any time in any of the different treatments (Table 4.4).

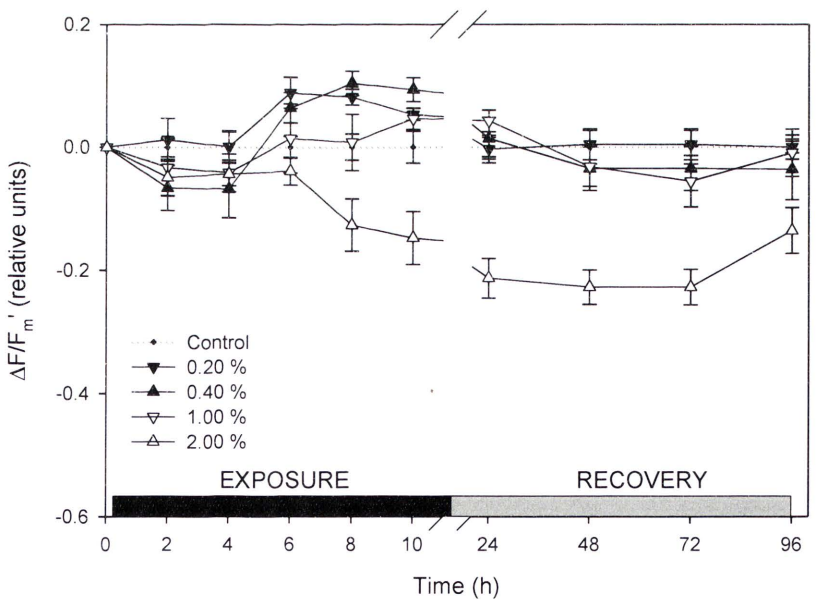

Figure 4.5: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of Tapis crude oil and Ardrox. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

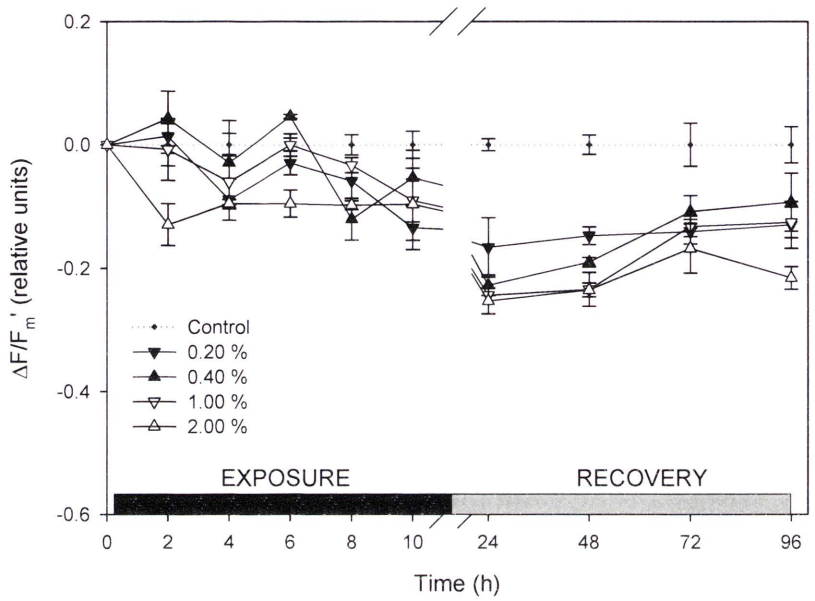

Figure 4.6: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of Corexit 9527. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

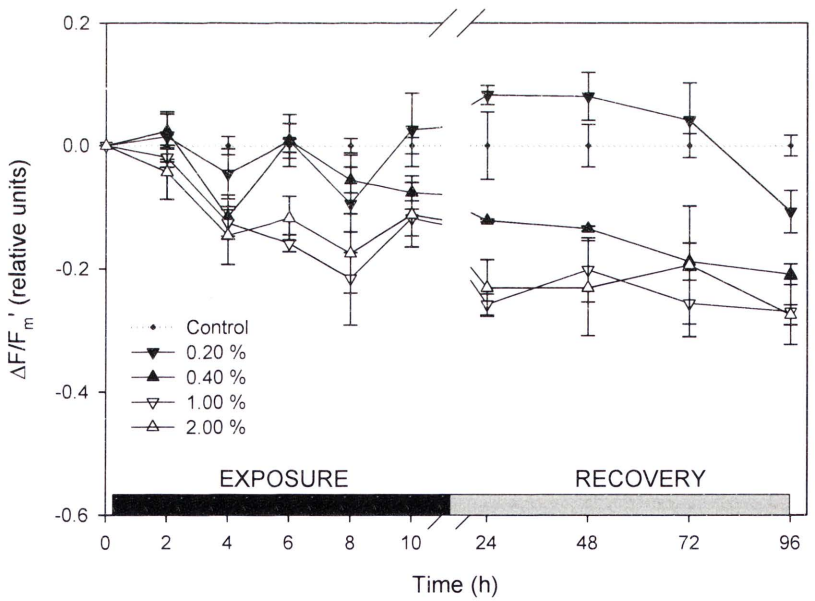

Figure 4.7: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of Ardrox. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$)

Table 4.2: Repeated measures ANOVA of the effective quantum yield of *Z. capricorni* exposed to the different concentrations of a) Tapis crude oil alone, b) Tapis crude oil + Corexit 9527, c) Tapis crude oil + Ardrox, Corexit 9527 alone and Ardrox alone. Degrees of freedom for interaction were exposure = 16, recovery = 12; for time effect exposure = 4, recovery = 3; and for concentration effect exposure = 4, recovery = 4. Values in bold denote a significant difference at P = 0.05.

Treatment	Exposure		Recovery	
	F	*P*	*F*	*P*
a. Tapis				
Concentration	0.470	0.757	1.407	0.237
Time	2.203	0.170	2.654	0.120
Concentration x Time	0.944	0.539	1.204	0.368
b. Tapis + C9527				
Concentration	3.564	**0.047**	0.654	0.637
Time	13.070	**0.002**	1.105	0.402
Concentration x Time	1.831	0.093	1.582	1.171
c.Tapis + Ardrox				
Concentration	2.905	**0.011**	11.883	**0.001**
Time	13.317	**0.002**	4.649	**0.037**
Concentration x Time	6.054	**0.010**	1.190	0.350
d. C9527				
Concentration	9.634	**0.002**	29.057	**0.000**
Time	4.022	0.053	12.124	**0.002**
Concentration x Time	2.490	**0.024**	1.175	0.359
e. Ardrox				
Concentration	9.486	**0.002**	14.405	**0.000**
Time	12.096	**0.003**	8.459	**0.007**
Concentration x Time	3.819	**0.002**	2.010	0.076

Table 4.3: One way analysis of variance (ANOVA) of the effective quantum yield of *Z. capricorni* exposed to the Tapis crude oil, Tapis crude oil + Corexit 9527 (C9527), Tapis crude oil + Ardrox, Corexit 9527 (C9527) alone and Ardrox alone treatments. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text. nc denotes ANOVA not calculated (no significant difference in the RmANOVA- Table 4.3). Values in bold denote a significant difference at P = 0.05.

Treatment		Exposure					Recovery			
		2	4	6	8	10	24	48	72	96
Tapis	*F*	nc	nc	nc	nc	nc	nc	nc	nc	nc
	P	nc	nc	nc	nc	nc	nc	nc	nc	nc
Tapis + C9527	*F*	2.759	0.228	9.250	3.576	0.991	nc	nc	nc	nc
	P	**0.008**	0.917	**0.048**	**0.002**	0.456	nc	nc	nc	nc
Tapis + Ardrox	*F*	0.900	1.309	5.813	8.793	27.091	7.653	11.218	10.687	2.498
	P	0.499	0.331	**0.011**	**0.003**	**0.000**	**0.004**	**0.001**	**0.001**	0.110
C9527	*F*	5.538	3.004	10.165	3.715	2.877	18.167	28.721	10.132	6.347
	P	**0.013**	0.072	**0.002**	**0.042**	0.080	**0.000**	**0.000**	**0.002**	**0.008**
Ardrox	*F*	1.246	6.311	8.114	5.618	4.660	19.028	6.771	4.643	12.958
	P	0.353	**0.008**	**0.003**	**0.012**	**0.022**	**0.000**	**0.007**	**0.022**	**0.001**

Table 4.4: One way analysis of variance (ANOVA) of chlorophyll *a* pigments in *Z. capricorni* at ten and 96 hours exposed to Tapis crude oil, Tapis crude oil + C 9527, Tapis crude oil + Ardrox, C 9527 alone and Ardrox alone. Values in bold denote significant differences at $p = 0.05$; values with same numbers are similar ($n = 3$).

	Treatment	0.00%	0.20%	0.40%	1.00%	2.00%	*F*	*P*
10h	Tapis	7.2 ± 0.5	9.8 ± 0.8	6.7 ± 0.3	6.3 ± 0.8	6.6 ± 0.9	3.420	0.081
	Tapis + C9527	11.1 ± 1.7	10.1 ± 1.4	11.4 ± 1.6	8.3 ± 2.1	11.9 ± 0.9	0.803	0.418
	Tapis + Ardrox	12.2 ± 2.7	10.9 ± 0.6	11.5 ± 0.5	11.9 ± 0.9	11.6 ± 0.2	0.116	0.348
	C9527	7.1 ± 1.3	10.1 ± 1.8	8.0 ± 1.5	8.9 ± 0.5	8.0 ± 0.3	0.827	0.502
	Ardrox	9.2 ± 0.4	10.4 ± 1.6	7.0 ± 1.2	8.0 ± 1.7	8.2 ± 0.4	1.076	0.281
96h	Tapis	9.3 ± 1.7	9.8 ± 0.8	11.2 ± 2.9	11.2 ± 1.0	7.1 ± 1.5	0.886	0.542
	Tapis + C9527	12.3 ± 3.2	13.4 ± 1.1	10.3 ± 1.0	9.8 ± 2.6	13.6 ± 1.0	0.731	0.614
	Tapis + Ardrox	12.2 ± 1.6	12.3 ± 1.0	12.5 ± 0.9	12.4 ± 0.9	13.0 ± 0.7	0.077	0.723
	C9527	7.8 ± 1.1	10.2 ± 1.5	7.6 ± 1.3	8.8 ± 0.6	7.7 ± 0.4	1.003	0.239
	Ardrox	12.2 ± 1.3	13.0 ± 0.8	10.1 ± 2.2	10.4 ± 0.5	9.3 ± 1.8	1.106	0.307

4.3.2.2 Halophila ovalis

Chlorophyll a fluorescence

The crude alone treatment resulted in no significant impact to *Halophila ovalis* over the exposure or recovery days (Figure 4.8; Table **4.5**). Although the 2.00 % concentration showed the greatest decrease in $\Delta F/F_m$' over the exposure day compared with the other concentrations this was never found to be significantly different in the repeated measures ANOVA. A one way ANOVA at two hours, however, did detect this concentration significantly lower than the control, 0.20 and 0.40 % concentrations (P = 0.044).

Both dispersed crude treatments led to an almost immediate, but short-lived impact to *H. ovalis*. The crude dispersed with Corexit 9527 treatment elicited a negative effect on the seagrass at two hours exposure with the 2.00 % concentration significantly lower than the control ($P < 0.01$). Both the 2.00 % and 1.00 % concentrations were lower than the elevated $\Delta F/F_m$' in the 0.20 and 0.40 % concentrations (Figure 4.9; Table 4.6). At ten hours exposure, all concentrations appeared slightly elevated in $\Delta F/F_m$' above the control, but no concentrations were significantly different. No effect was evident to the seagrass over the recovery period (Table 4.5).

Similarly, the Ardrox dispersed crude treatments (Figure 4.10) led to an impact to *H. ovalis* at two hours exposure, but no impacts after four hours in either the exposure or recovery days (Table 4.5; Table 4.6). At two hours exposure, the $\Delta F/F_m$' of the seagrass exposed to the 2.00 % concentration decreased significantly below all concentrations (One way ANOVA, Table 4.6). By four hours the $\Delta F/F_m$' in this largest concentration had recovered somewhat and was only significantly different to the 0.20 % and 0.40 % concentrations which were slightly elevated above the control. The 0.20 % and 0.40 % were never significantly different to the control. Again, similar to the Corexit 9527 dispersed treatment, there was no significant impact detected over the recovery days.

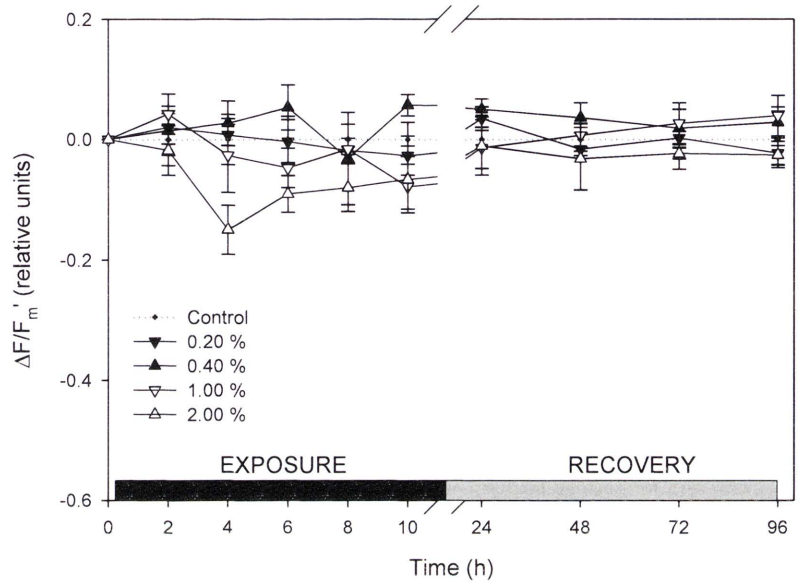

Figure 4.8: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of Tapis crude oil. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

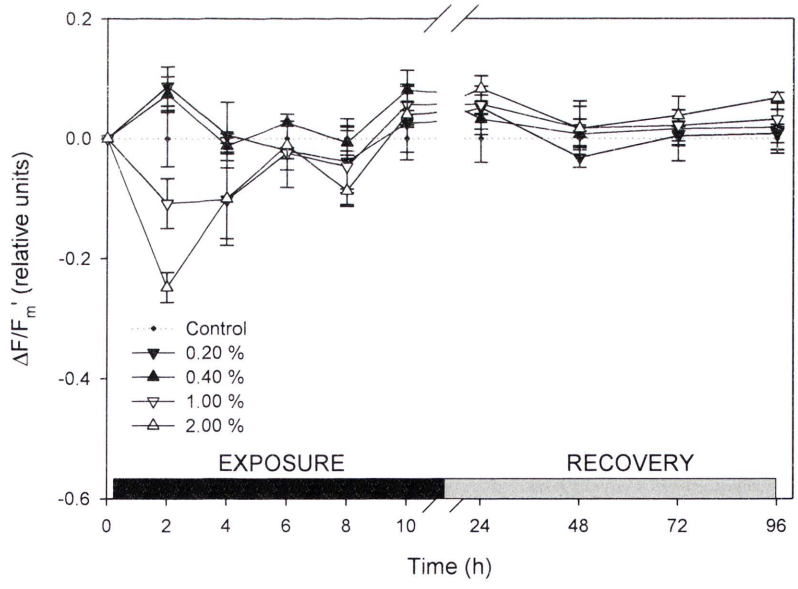

Figure 4.9: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of Tapis crude oil and Corexit 9527. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

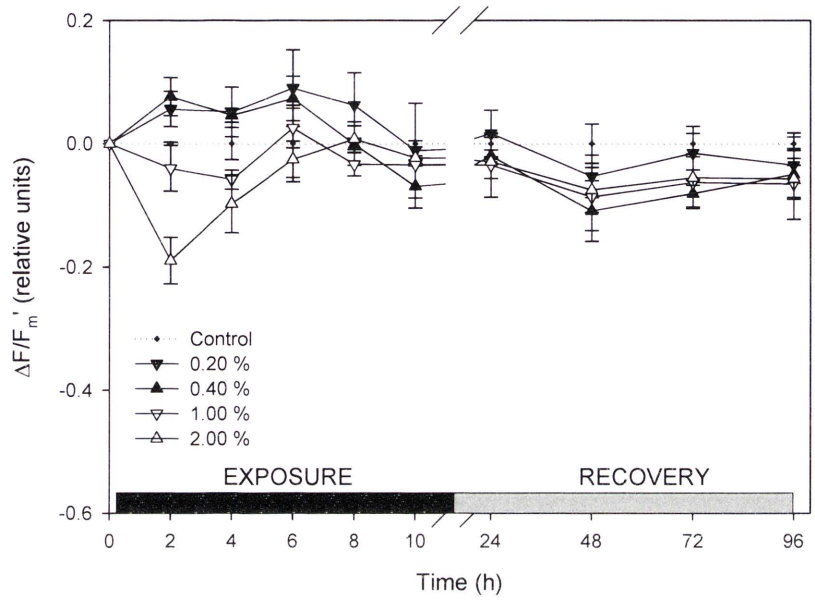

Figure 4.10: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of Tapis crude oil and Ardrox. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

There was a variable response in $\Delta F/F_m'$ of *H. ovalis* to the Corexit 9527 alone WAF treatment even after the removal of the chambers (Figure 4.11). Minimal impact and noticeably fluctuating values were evident in this treatment. There was a significant time effect with the last two exposure day measurements differing significantly to the earlier exposure day measurements (Table 4.5). This time effect was enhanced by the 0.40 % concentration being significantly lower than the 2.00 % concentration at six hours (Table 4.6). The recovery day measurements were also quite variable and no significant effects were detected in the repeated measures ANOVA (Table 4.5). At no time did any concentration decrease more than 0.2 units below the control. Differences were detected in the one way ANOVAs at 72 hours, so the lack of significant effects in the main ANOVA are probably due to the inherent variability in the data.

The Ardrox alone WAF treatment led to significant impacts to *H. ovalis* over the exposure and recovery days (Figure 4.12; Table 4.5). There were fluctuations in the response of the seagrass from the different concentrations but overall, there was a highly

significant concentration effect ($P < 0.001$) over the exposure day (Table 4.5). At two hours, the 2.00 % significantly less than the control, 0.20 and 2.00 % ($P < 0.01$; Table 4.6). At four hours, only the 1.00 % concentration was significantly less than the control (Table 4.6). At ten hours, the 0.40 % concentration was significantly different to the 1.00 % and 2.00 % WAF concentrations. An increase in impact to the seagrass occurred following the removal of the chambers (Figure 4.12) and a highly significant concentration effect was detected ($P < 0.001$; Table 4.5). The $\Delta F/F_m'$ of the seagrass exposed to the 2.00 % concentration was significantly lower than the control at all times with all measurements greater than 0.25 units below the control (Table 4.6). No other concentration fell below 0.2 units below the control and none differed to the control.

Photosynthetic Pigment Analysis

Few differences were detected in the chlorophyll *a* pigment analyses, at 10 or 96 hours post exposure (Table 4.7) of the crude alone. The chlorophyll a concentration in the 0.20, 0.40 and 2.00 % WAF concentrations of the Ardrox dispersed crude differed to the control at ten hours (Table 4.7).

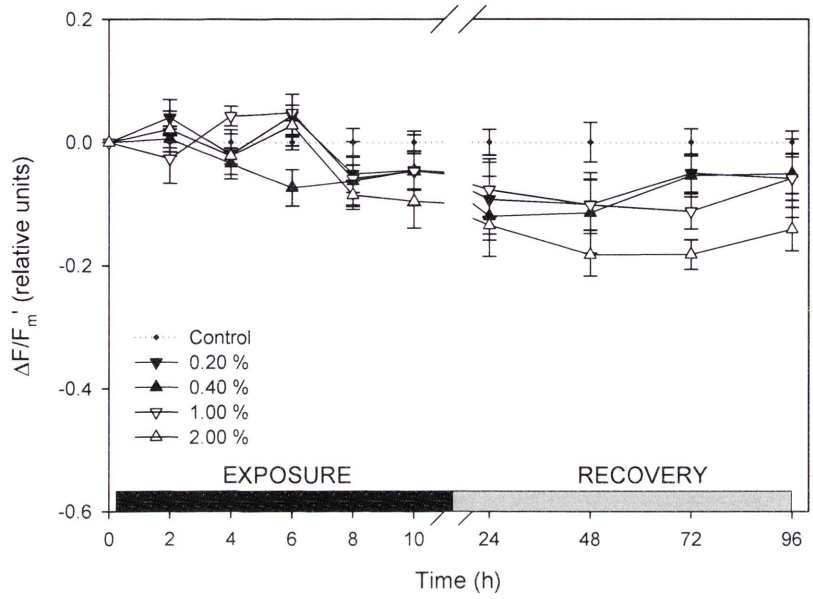

Figure 4.11: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of Corexit 9527. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

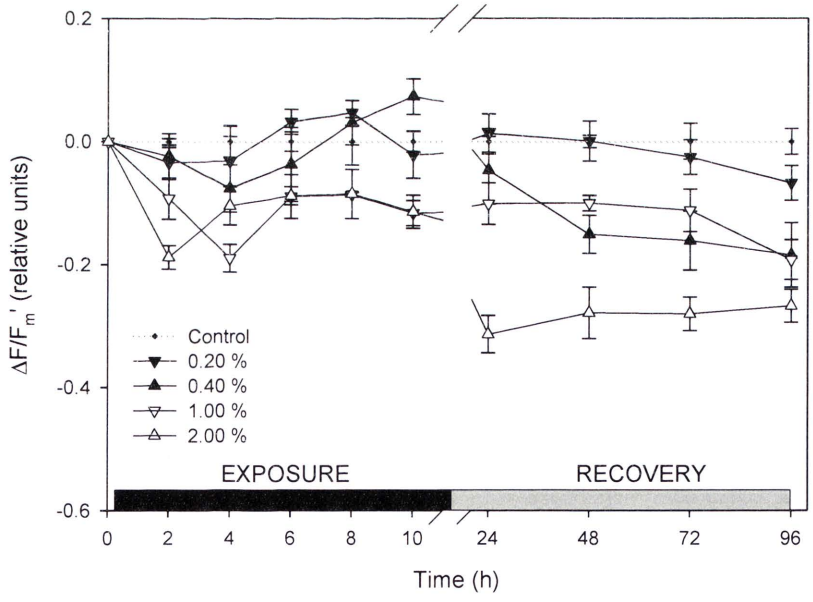

Figure 4.12: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of Ardrox. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

Table 4.5: Repeated measures ANOVA of the effective quantum yield of *H. ovalis* exposed to the different concentrations of a) Tapis crude oil alone, b) Tapis crude oil + Corexit 9527, c) Tapis crude oil + Ardrox, Corexit 9527 alone and Ardrox alone. Degrees of freedom for interaction were exposure = 16, recovery = 12; for time effect exposure = 4, recovery = 3; and for concentration effect exposure = 4, recovery = 4. Values in bold denote a significant difference at P = 0.05.

Treatment	Exposure		Recovery	
	F	*P*	*F*	*P*
a. Tapis				
Concentration	2.536	0.106	0.657	0.636
Time	1.990	0.201	0.138	0.934
Concentration x Time	1.783	0.103	1.254	0.312
b. Tapis + C9527				
Concentration	2.407	0.119	0.887	0.506
Time	14.074	**0.002**	2.626	0.122
Concentration x Time	3.867	**0.002**	1.266	0.305
c. Tapis + Ardrox				
Concentration	6.298	**0.008**	1.097	0.410
Time	7.903	**0.010**	3.770	0.059
Concentration x Time	2.160	**0.047**	0.421	0.938
d. C9527				
Concentration	1.432	0.293	2.233	0.138
Time	7.352	**0.012**	2.073	0.100
Concentration x Time	1.045	0.138	1.182	0.354
e. Ardrox				
Concentration	13.423	**0.000**	31.704	**0.000**
Time	4.098	0.051	1.467	0.295
Concentration x Time	2.162	**0.047**	0.760	0.682

Table 4.6: One way analysis of variance (ANOVA) of the effective quantum yield of *H. ovalis* exposed to the Tapis crude oil, Tapis crude oil + Corexit 9527 (C9527), Tapis crude oil + Ardrox, Corexit 9527 (C9527) alone and Ardrox alone treatments. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text. nc denotes ANOVA not calculated (no significant difference in the RmANOVA- Table 4.3). Values in bold denote a significant difference at P = 0.05.

Treatment		Exposure					Recovery			
		2	4	6	8	10	24	48	72	96
Tapis	*F*	nc	nc	nc	nc	nc	nc	nc	nc	nc
	P	nc	nc	nc	nc	nc	nc	nc	nc	nc
Tapis + C9527	*F*	25.811	1.588	0.361	0.593	0.750	nc	nc	nc	nc
	P	**0.000**	0.252	0.831	0.676	0.580	nc	nc	nc	nc
Tapis + Ardrox	*F*	17.190	4.263	2.043	1.342	0.616	nc	nc	nc	nc
	P	**0.000**	**0.029**	0.164	0.320	0.661	nc	nc	nc	nc
C9527	*F*	0.613	1.796	4.465	1.249	0.722	1.161	2.048	6.055	1.621
	P	0.663	0.206	**0.025**	0.352	0.596	0.384	0.163	**0.010**	0.244
Ardrox	*F*	8.416	6.502	2.473	4.321	8.569	15.748	15.764	10.931	9.084
	P	**0.003**	**0.008**	0.112	**0.028**	**0.003**	**0.000**	**0.000**	**0.001**	**0.002**

Table 4.7: One way analysis of variance (ANOVA) of chlorophyll *a* pigments in *H. ovalis* at ten and 96 hours exposed to Tapis crude oil, Tapis crude oil + C9527, Tapis crude oil + Ardrox, C9527 alone and Ardrox alone. Values in bold denote significant differences at $p = 0.05$; values with same numbers are similar (n = 3).

	Treatment	0.00%	0.20%	0.40%	1.00%	2.00%	F	P
10 h	Tapis	13.2 ± 0.3	13.6 ± 0.5	14.7 ± 0.9	14.1 ± 1.4	13.4 ± 0.8	0.426	0.632
	Tapis + C9527	13.1 ± 1.0	14.0 ± 1.6	13.4 ± 0.3	12.2 ± 1.0	11.9 ± 0.7	0.751	0.415
	Tapis + Ardrox	**9.7 ± 0.6^{a}**	**12.9 ± 1.0^{ab}**	**15.1 ± 1.0^{b}**	**9.6 ± 1.5^{a}**	**15.4 ± 1.2^{b}**	**6.402**	**0.036**
	C9527	12.3 ± 1.4	11.9 ± 1.2	10.1 ± 1.2	8.6 ± 0.5	13.6 ± 2.0	2.117	0.163
	Ardrox	11.4 ± 2.3	9.8 ± 0.9	12.1 ± 0.6	15.3 ± 2.6	10.4 ± 2.3	1.265	0.091
96 h	Tapis	11.5 ± 1.5	11.7 ± 0.6	15.2 ± 1.0	13.6 ± 2.7	15.5 ± 1.3	1.350	0.271
	Tapis + C9527	12.9 ± 1.7	14.1 ± 1.6	13.7 ± 0.2	13.0 ± 0.4	10.7 ± 1.3	0.140	0.423
	Tapis + Ardrox	10.2 ± 3.3	10.3 ± 0.4	15.3 ± 0.8	10.8 ± 1.6	10.8 ± 1.6	1.396	0.393
	C9527	12.4 ± 1.2	12.7 ± 1.40	11.8 ± 3.3	12.6 ± 0.9	11.0 ± 2.4	0.480	0.425
	Ardrox	10.5 ± 3.3	12.6 ± 1.64	10.4 ± 2.9	10.9 ± 3.3	13.7 ± 2.4	0.210	0.131

4.3.3 IFO-380: non-dispersed, dispersed and dispersant alone

Total petroleum hydrocarbon (TPH) concentration

Most concentrations in the IFO-380 alone and IFO-380 dispersed treatments showed a significant decrease in the total petroleum hydrocarbon concentration over the ten hour exposure period (Figure 4.13). For the IFO-380 alone treatments (Figure 4.13), all concentrations except the 1.00 % concentration decreased significantly below the pre-exposure TPH concentration (Table 4.8). The 2.00 % displayed the greatest decrease in TPH, about 60 % of the initial TPH concentration. The dispersed IFO-380 treatments lost a large portion of TPH over the exposure period with an increasing loss evident in the larger WAF concentrations. Both the Slickgone dispersed IFO-380 (Figure 4.13) and the Corexit dispersed IFO-380 (Figure 4.13) showed significant losses in all concentrations after ten hours exposure. There was slightly less TPH remaining in the IFO-380 and Slickgone treatments with only 25 % remaining in the 2.00 % WAF treatment and 29 % in the corresponding IFO-380 and Corexit 9500 WAF treatment.

Some loss of TPH was evident in the Slickgone alone WAF treatments (Figure 4.13) with the 0.40 % and 1.00 % WAF concentrations significantly below the initial exposure measurement. The 0.20 % had decreased to almost half, 55 %, of the pre-exposure concentration. The Corexit alone WAF treatment only showed significant differences due to an increase in the TPH value following the exposure period in the 0.20 and 0.40 % WAF concentrations (Figure 4.13; Table 4.8).

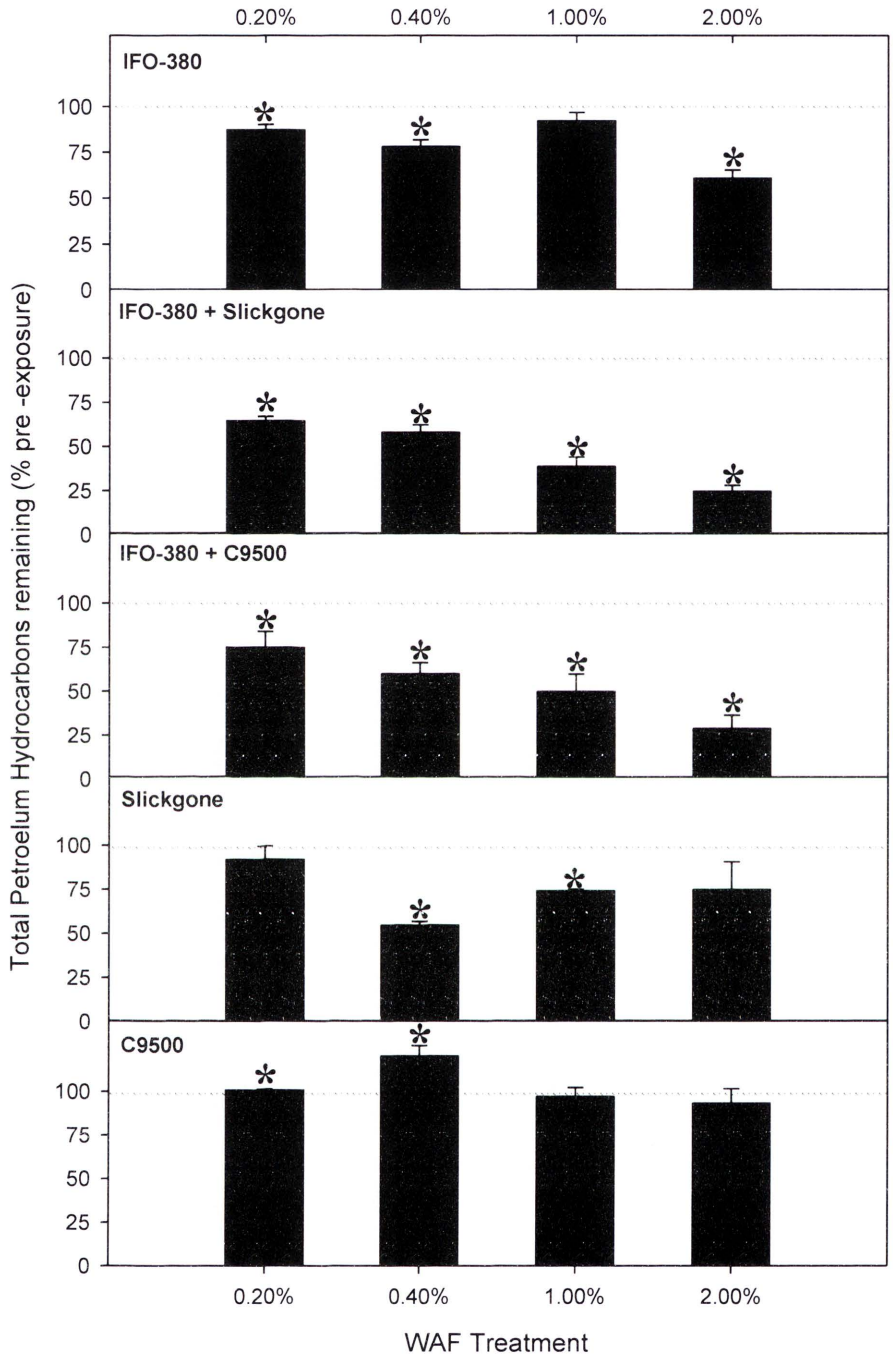

Figure 4.13: Percentage TPH remaining after ten hours exposure determined by UV fluorescence for IFO-380, IFO-380 + Slickgone; IFO-380 + Corexit 9500 (C9500); Slickgone and Corexit 9500 WAF treatments (* denotes a significant difference between the pre- and post concentrations Average ± standard error of the mean are shown (n = 3).

Table 4.8 Independent *t* test analysis of the total hydrocarbon concentration following ten hours exposure of IFO-380 alone; IFO-380 + Slickgone; IFO-380 + Corexit 9500 (C9500); Slickgone and Corexit 9500 (C9500) (n = 3). Values in bold denote a significant difference at P = 0.05.

Treatment	Concentration							
	0.20 %		*0.40 %*		*1.00 %*		*2.00 %*	
	t	*p*	*t*	*p*	*t*	*p*	*t*	*p*
IFO-380	3.38	**0.028**	3.33	**0.029**	1.17	0.307	5.72	**0.005**
IFO-380 + Slickgone	10.07	**0.001**	21.80	**0.000**	28.32	**0.000**	15.90	**0.004**
IFO-380 + C9500	15.75	**0.000**	18.44	**0.000**	24.34	**0.000**	14.54	**0.000**
Slickgone alone	0.61	0.575	17.76	**0.000**	19.93	**0.000**	0.99	0.378
C9500 alone	4.18	**0.014**	4.32	**0.012**	0.55	0.613	0.01	0.990

4.3.3.1 Zostera capricorni

Chlorophyll a fluorescence

The IFO-380 elicited no detectable impact from *Zostera capricorni* over the ten hours exposure day (Figure 4.14; Table 4.9). There was only minimal impact on the seagrass over the recovery days. The last two days of measurements were elevated slightly above those of the first two days of measurements; at 48 hours the $\Delta F/F_m'$ of *Z. capricorni* exposed to the 0.40 % WAF was significantly lower than that of the 2.00 % WAF whilst at 72 hours it was lower than the 1.00 % WAF concentration. No concentration differed from the control at any time and there were no impacts detected during the final measurement day, 96 hours (Table 4.10).

The two dispersed IFO-380 treatments (Figure 4.15 Figure 4.15) resulted in very different impacts to *Z. capricorni*. The Slickgone dispersed IFO-380 treatment showed no impact throughout the entire experiment, while Corexit 9500 dispersed IFO-380 showed significant impacts during the exposure and recovery period (Table 4.9). *Zostera capricorni* exposed to the IFO-380 dispersed with Corexit 9500 treatment began to show significant effects at six hours exposure (Figure 4.16; Table 4.9). There

was a significant concentration effect overall ($P < 0.01$; Table 4.9) with the 1.00 and 2.00 % treatments being different to the control ($P < 0.05$). One way ANOVAs detected the $\Delta F/F_m'$ of the seagrass exposed to the 2.00 % WAF as different from the control at six hours, at eight hours for the 1.00 % WAF, whilst at ten hours both concentrations were different compared to the control (Table 4.10). No other concentrations differed to each other. There was a significant time effect ($P < 0.01$) due to the low $\Delta F/F_m'$ in the two higher concentrations at ten hours. Seagrass showed increasing recovery with each successive day of measurement following the removal of the chambers (Figure 4.16; Table 4.9). An interaction effect ($P < 0.01$; Table 4.9) was evident in this successive recovery. At 24 hours, the $\Delta F/F_m'$ of *Z. capricorni* exposed to the 0.40, 1.00 and 2.00 % WAF concentrations were significantly lower than the control. At 48 hours, only the 2.00 % WAF still differed in $\Delta F/F_m'$ from the control and no concentrations differed after 72 or 96 hours post exposure (Table 4.10).

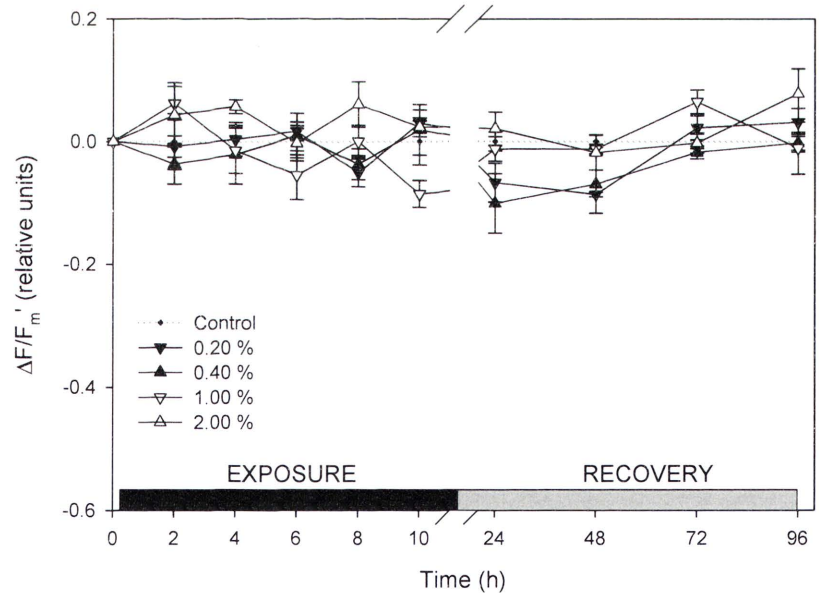

Figure 4.14: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of IFO-380. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

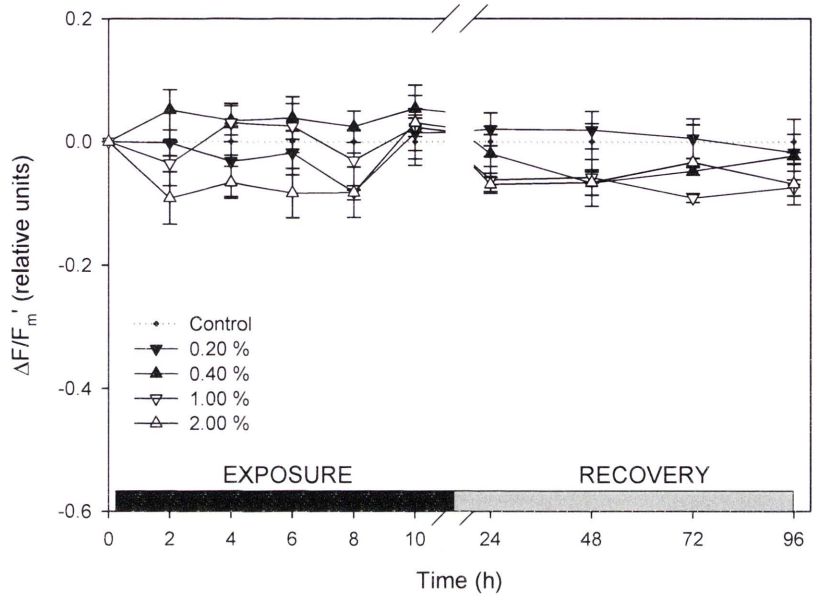

Figure 4.15: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of IFO-380 and Slickgone. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

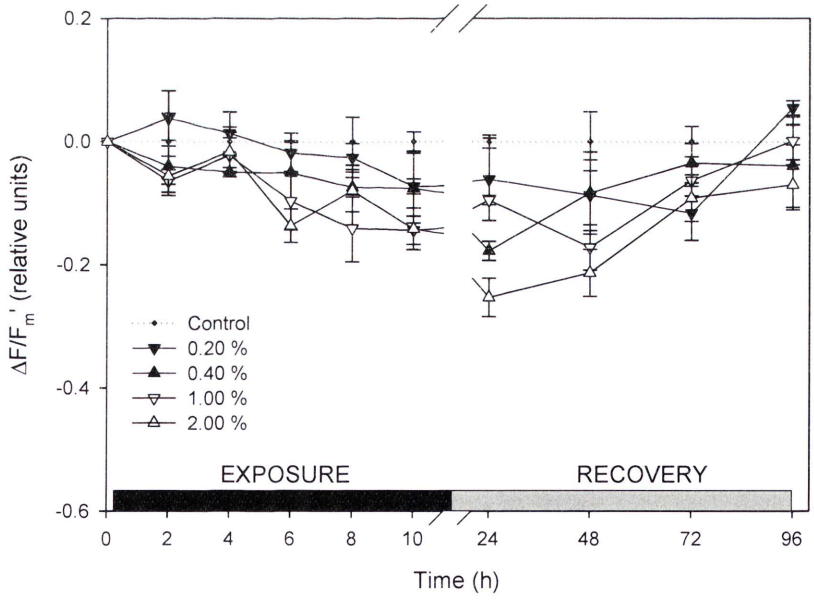

Figure 4.16: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of IFO-380 and Corexit 9500. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

Similar to the two dispersed IFO-380 treatments, the two dispersant alone treatments (Figure 4.17 &Figure 4.18) showed different results. The Slickgone alone treatment resulted in no detectable impact to *Z. capricorni* throughout the exposure or recovery periods (Fig 4.16; Table 4.9). Conversely, the Corexit 9500 alone treatment negatively impacted the $\Delta F/F_m'$ of *Z. capricorni* during the exposure period continuing through the first few days of recovery (Figure 4.18). Over the exposure day there were significant time and concentration effects (Table 4.9). One way ANOVAs did not detect differences at any particular time, but the overall concentration effect (P = 0.045) showed the 2.00 % to be less than the control (P = 0.042). An interaction effect over the recovery period was due to the first two days of measurements showing highly significant negative impacts to the seagrass and the last two days showing no effects.

Chlorophyll a pigment concentrations

The chlorophyll *a* pigment analysis detected only one significant difference to *Z. capricorni* following exposure to the IFO-380, dispersed IFO-380 or the dispersants alone treatments (Table 4.11). This single significant difference was detected in the Corexit 9500 alone treatment at 96 hours (Table 4.11). For this treatment the 0.40 % concentration differed from the 2.00 % concentration (Table 4.11).

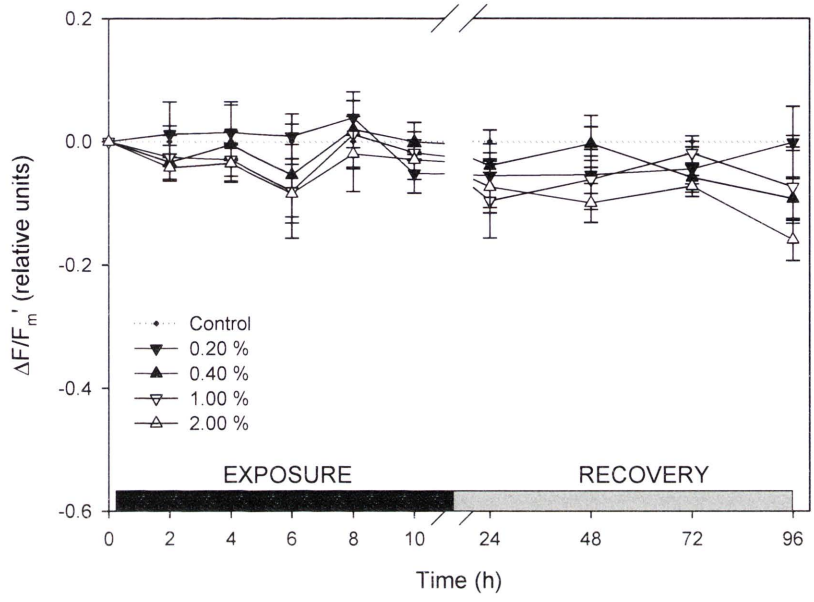

Figure 4.17: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of Slickgone. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

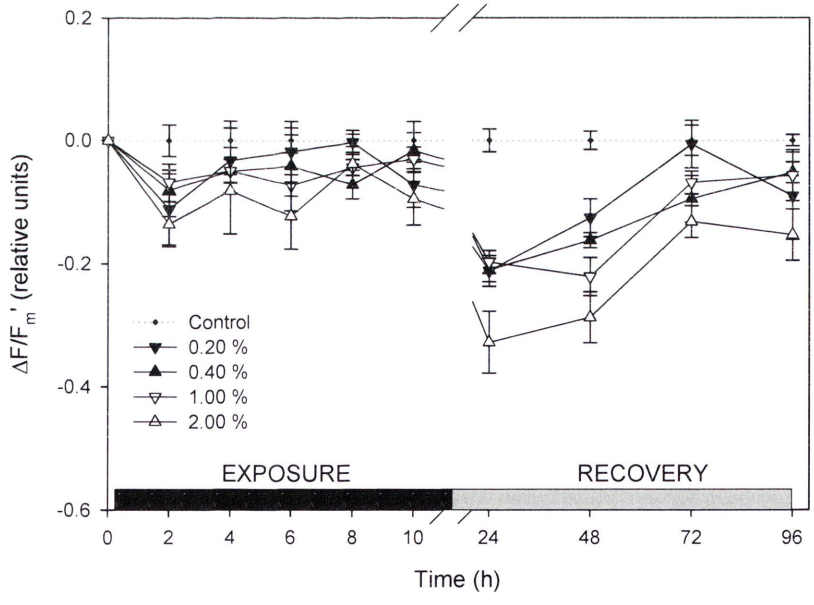

Figure 4.18: Change in effective quantum yield of *Z. capricorni* exposed to different concentrations of the water soluble fraction of Corexit 9500. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3)

Table 4.9: Repeated measures ANOVA of the effective quantum yield of *Z. capricorni* exposed to different concentrations of a) IFO-380 alone, b) IFO-380 + Slickgone, c) IFO-380 + Corexit 9500 (C9500), Slickgone alone and Corexit 9500 (C9500) alone. Degrees of freedom for interaction were exposure = 16, recovery = 12; for time effect exposure = 4, recovery = 3; and for concentration effect exposure = 4, recovery = 4. Values in bold denote a significant difference at P = 0.05.

Treatment	Exposure		Recovery	
	F	*P*	*F*	*P*
a. IFO-380				
Concentration	1.117	0.401	2.875	**0.016**
Time	0.501	0.737	10.317	**0.004**
Concentration x Time	1.764	0.107	2.726	0.090
b. IFO-380 + Slickgone				
Concentration	2.529	0.107	2.041	0.164
Time	2.045	0.192	0.464	0.715
Concentration x Time	0.826	0.648	2.135	0.061
c. IFO-380 + C9500				
Concentration	6.350	**0.008**	7.453	**0.005**
Time	8.982	**0.007**	12.386	**0.002**
Concentration x Time	1.230	0.320	3.220	**0.009**
d. Slickgone				
Concentration	0.656	0.636	2.527	0.107
Time	2.461	0.141	0.646	0.607
Concentration x Time	0.661	0.800	1.256	0.311
e. C9500				
Concentration	3.427	**0.045**	25.587	**0.000**
Time	4.612	**0.039**	22.150	**0.000**
Concentration x Time	0.950	0.533	2.172	0.057

Table 4.10: One way analysis of variance (ANOVA) of the effective quantum yield of *Z. capricorni* exposed to the IFO-380, IFO-380 + Slickgone, IFO-380 + Corexit 9500 (C9500), Slickgone alone and Corexit 9500 (C9500) alone treatments. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text. nc denotes ANOVA not calculated (no significant difference in the RmANOVA- Table 4.3). Values in bold denote a significant difference at P = 0.05.

Treatment		Exposure					Recovery			
		2	4	6	8	10	24	48	72	96
IFO-380	*F*	nc	nc	nc	nc	nc	5.216	2.250	3.953	1.470
	P	nc	nc	nc	nc	nc	**0.016**	0.136	**0.035**	0.282
IFO-380 + Slickgone	*F*	nc	nc	nc	nc	nc	nc	nc	nc	nc
	P	nc	nc	nc	nc	nc	nc	nc	nc	nc
IFO-380 + C9500	*F*	1.646	0.813	3.859	4.199	7.761	8.474	4.365	2.386	1.386
	P	0.238	0.545	**0.038**	**0.030**	**0.004**	**0.003**	**0.027**	0.121	0.307
Slickgone	*F*	nc	nc	nc	nc	nc	nc	nc	nc	nc
	P	nc	nc	nc	nc	nc	nc	nc	nc	nc
C9500	*F*	1.763	0.392	2.022	2.885	2.280	22.844	13.014	3.511	1.801
	P	0.213	0.810	0.167	0.079	0.132	**0.000**	**0.001**	**0.049**	0.205

Table 4.11 One way analysis of variance (ANOVA) of chlorophyll *a* pigments in *Z. capricorni* at ten and 96 hours exposed to IFO-380, IFO-380 + Slickgone, IFO-380 + C9500, Slickgone alone and C9500 alone. Values in bold denote significant differences at P = 0.05 (n = 3).

	Treatment	0.00%	0.20%	0.40%	1.00%	2.00%	*F*	*P*
10 h	IFO-380	13.4 ± 2.4	12.2 ± 0.6	11.3 ± 1.0	10.5 ± 0.8	13.7 ± 2.5	0.556	0.412
	IFO-380 + Slickgone	15.8 ± 1.5	13.0 ± 3.5	11.2 ± 2.7	10.9 ± 2.5	12.2 ± 1.3	0.447	0.568
	IFO-380 + C9500	13.0 ± 2.4	12.8 ± 1.3	12.7 ± 1.2	12.7 ± 1.3	13.3 ± 1.7	0.019	0.701
	Slickgone	10.4 ± 0.6	10.7 ± 2.1	8.0 ± 1.1	6.9 ± 0.5	5.9 ± 0.5	1.420	0.258
	C9500	10.5 ± 0.6	11.3 ± 2.4	13.3 ± 3.4	10.5 ± 2.8	5.8 ± 0.5	0.019	0.214
96 h	IFO-380	11.3 ± 1.3	10.8 ± 0.6	11.5 ± 1.0	12.5 ± 3.1	13.7 ± 2.5	0.342	0.104
	IFO-380 + Slickgone	11.5 ± 1.1	13.6 ± 1.3	12.5 ± 1.7	11.7 ± 1.6	9.7 ± 0.0	1.124	0.270
	IFO-380 + C9500	13.9 ± 1.4	14.5 ± 1.2	13.1 ± 2.2	13.3 ± 2.3	11.7 ± 2.3	0.277	0.212
	Slickgone	9.4 ± 1.6	8.4 ± 1.3	6.9 ± 0.8	6.5 ± 1.1	5.9 ± 0.5	3.559	0.341
	C9500	**10.7 ± 0.8^{ab}**	**9.7 ± 1.6^{ab}**	**12.6 ± 1.8^{a}**	**9.3 ± 1.7^{ab}**	**5.2 ± 0.7^{b}**	**0.277**	**0.041**

4.3.3.2 Halophila ovalis

Chlorophyll a fluorescence

The IFO-380 alone treatment led to decreases in the $\Delta F/F_m'$ of *H. ovalis* up to six hours exposure (Figure 4.19; Table 4.12). The 2.00 % WAF concentration led to a rapid and significant decrease to the $\Delta F/F_m'$ of the seagrass at two and four hours exposure (Table 4.13). At four hours, the $\Delta F/F_m'$ of *H. ovalis* exposed to the 0.20 and 1.00 % WAF concentrations were also significantly less than the control. There were no detectable impacts over the recovery day (Table 4.12).

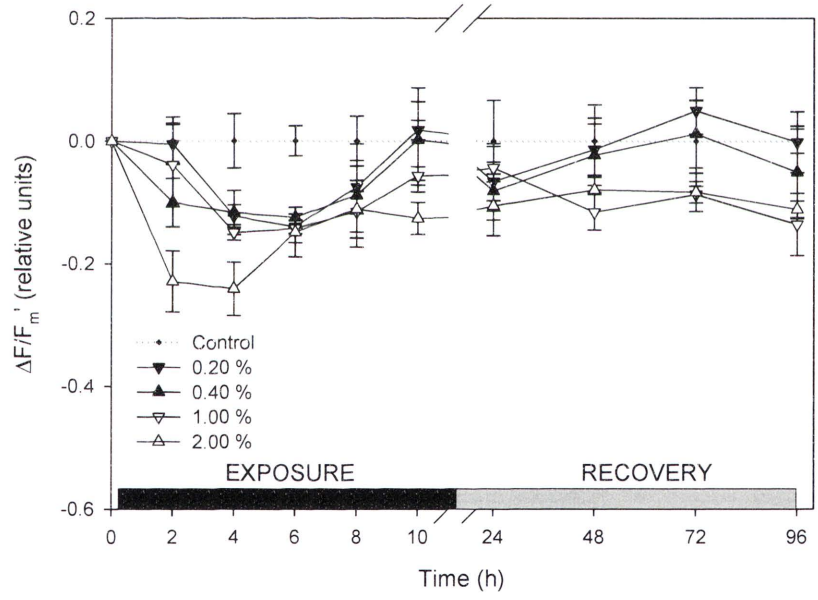

Figure 4.19: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of IFO-380. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

The dispersed IFO-380 treatments elicited significant responses from *H. ovalis* for both types of dispersants. The IFO-380 dispersed with Slickgone treatments resulted in highly significant interaction and main effects (Figure 4.20; Table 4.12). Overall, the 2.00 % was significantly lower than all other concentrations ($P<0.05$; pooled times across concentrations). One way ANOVAs found the 2.00 % WAF to be different to the

control at four, six and eight hours exposure. At two hours, the 2.00 % WAF was also significantly different to the 0.20 % WAF, whilst at six and eight hours was significantly different to all concentrations ($P < 0.05$). No differences were detected at ten hours exposure (Table 4.13). There was also a significant interaction effect during the recovery days. Overall, the 1.00 and 2.00 % WAF concentrations were significantly lower than the $\Delta F/F_m'$ of the control and the 0.20 % WAF concentration ($P < 0.05$). One way ANOVAs (Table 4.13) detected differences at 24 hours with the $\Delta F/F_m'$ of the seagrass exposed to the 1.00 and 2.00 % WAF concentrations, significantly less than the control and the 0.20 % WAF ($P < 0.01$). At 96 hours, the 1.00 % WAF was different to the control and the 0.20 % WAF concentration ($P < 0.05$). The time effect overall found the 24 hour measurement to be significantly less than the $\Delta F/F_m'$ in the 48 and 72 hour measurements due to the significant differences between concentrations.

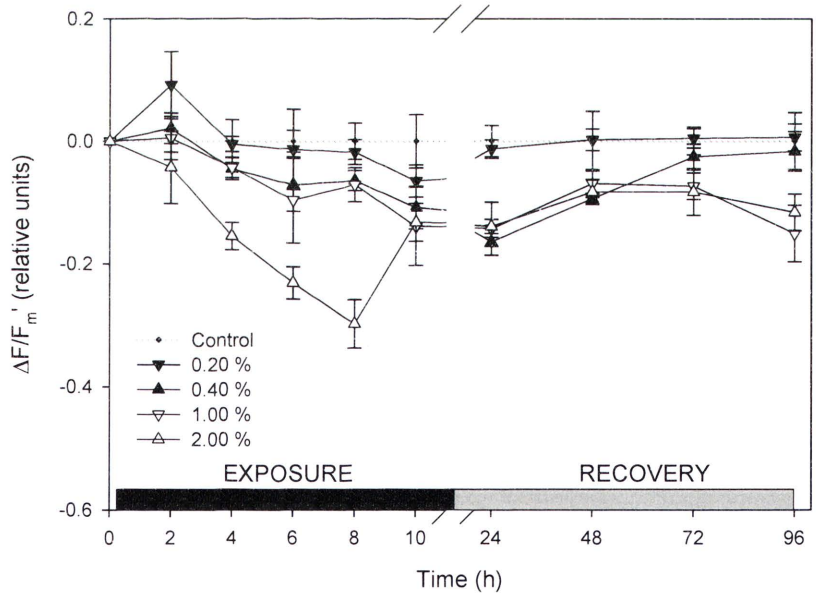

Figure 4.20: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of IFO-380 and Slickgone. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

The IFO-380 dispersed with Corexit 9500 treatment (Figure 4.21) resulted in significant differences during the exposure day; with a highly significant interaction effect ($P < 0.01$; Table 4.12). One way ANOVAs (Table 4.13) found significant differences

between concentrations at all times during the exposure day. The 2.00 % WAF concentration was significantly less than the control during the first four hours of exposure and also at ten hours ($P < 0.05$). At six hours, the 1.00 % WAF was significantly less than the control. During the recovery period there was a significant effect of time. This was likely enhanced by the severe depression of the 2.00 % WAF at 24 hours. At 24 hours, the 2.00 % WAF was significantly lower than the control, and the 0.20 and 0.40 % WAF concentrations ($P < 0.05$). Following this, there were no further detectable impacts.

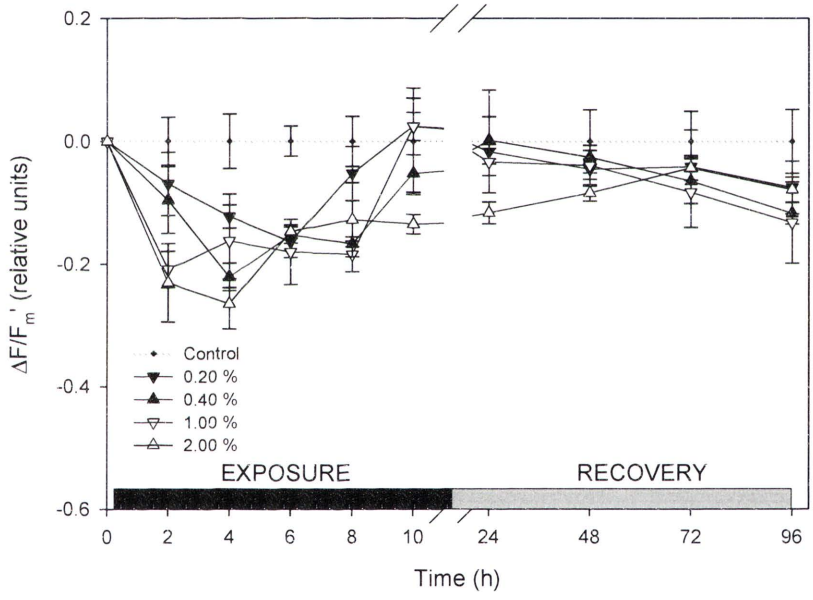

Figure 4.21: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of IFO-380 and Corexit 9500. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

The Slickgone alone treatment (Figure 4.22) led to significant interaction and main effects on the $\Delta F/F_m'$ of *H. ovalis* (Table 4.12). There was variability in the response of the seagrass over time between concentrations, but the 2.00 % was significantly different overall to the control. One way ANOVAs found significant difference only at eight hours with the 2.00 % differing to the control and the 1.00 %. The 0.40 % concentration was also significantly less than the control at this time. The seagrass showed signs of recovery with the replenishment of fresh seawater. There was only a

significant effect of time ($P < 0.010$) with a decrease in the measurements at 72 hours. No concentrations were significantly different at any time.

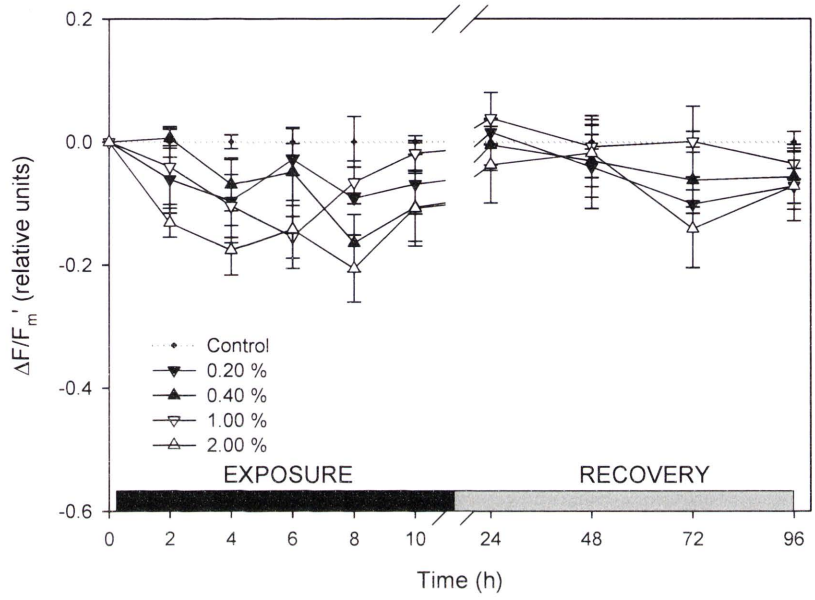

Figure 4.22: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of Slickgone Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

The largest response of any treatment occurred with exposure to the Corexit 9500 treatment (Figure 4.23; Table 4.12). Overall, the 2.00 % WAF was significantly less than all other concentrations ($P < 0.001$). One way ANOVAs (Table 4.13) detected the 2.00 % WAF to be significantly different to all other concentrations at most sampling times and was highly significantly different to the control ($P < 0.01$) at all times. At eight hours exposure, the 2.00 % WAF concentration was similar to the 0.40 % concentration. Other than the 2.00 % WAF concentration, no other treatments differed to each other during the exposure day. The seagrass showed no signs of recovery following the replenishment of fresh seawater over the recovery period (Figure 4.23). There was a significant interaction effect ($P = 0.046$) and highly significant concentration effect (Table 4.12). Overall, the 2.00 % was significantly less than all other concentrations. The 2.00 % WAF was different to the control at 24, 72 and 96 hours. The 2.00 % WAF differed to the 0.20 % WAF at all times of recovery and at 72 hours was different to all concentrations. At 24 hours, the 2.00 % WAF was different to

the control and 0.20 %WAF, at 48 hours only different to 0.20 % WAF, at 72 hours, different to all and 96 hours different to all except the 1.00 % WAF. No other treatment differed to the control or to each other.

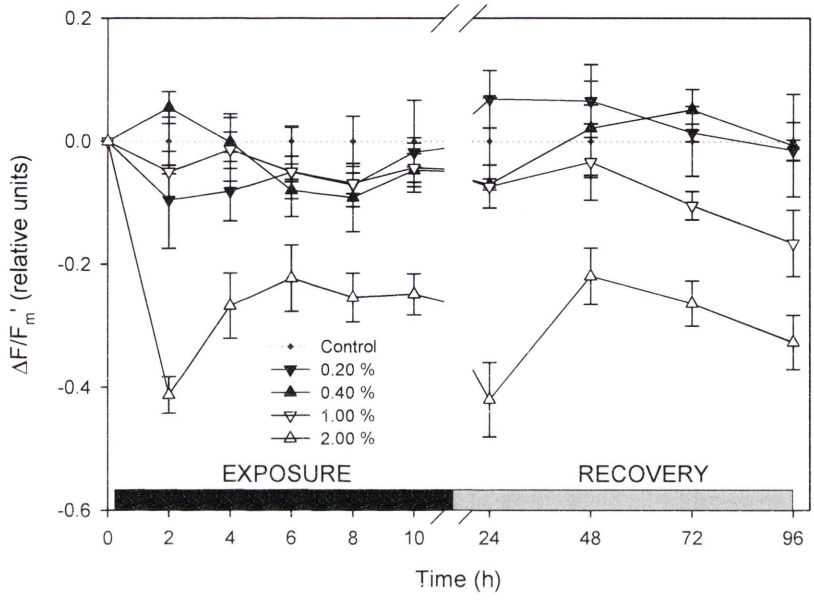

Figure 4.23: Change in effective quantum yield of *H. ovalis* exposed to different concentrations of the water soluble fraction of Corexit 9500. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

Chlorophyll a pigment concentrations

The chlorophyll *a* pigment concentrations of *H.ovalis* exposed to the IFO-380 alone treatments were significantly lower than the control at 96 hours for all treatment concentrations. The IFO-380 dispersed with Corexit 9500 displayed a significant difference between the control and the 2.00 % WAF concentration, whilst the control differed from both the 1.00 % and 2.00 % WAF concentrations in the Corexit 9500 alone treatment (Table 4.14). No differences were detected at 10 hours exposure (Table 14) in any of the treatments.

Table 4.12: Repeated measures ANOVA of the effective quantum yield of *Z. capricorni* exposed to the different concentrations of a) IFO-380 alone, b) IFO-380 + Slickgone, c) IFO-380 + C9500, Slickgone alone and C9500 alone. Degrees of freedom for interaction: exposure = 16, recovery = 12; for time effect exposure = 4, recovery = 3; and for concentration effect exposure = 4, recovery = 4. Values in bold denote a significant difference at P = 0.05.

Treatment	Exposure		Recovery	
	F	*P*	*F*	*P*
a. IFO-380				
Concentration	6.127	**0.009**	1.501	0.274
Time	13.746	**0.002**	2.223	0.163
Concentration x Time	2.404	**0.029**	1.617	0.160
b. IFO-380 + Slickgone				
Concentration	12.818	**0.001**	7.475	**0.005**
Time	19.971	**0.001**	21.680	**0.000**
Concentration x Time	3.273	**0.005**	2.847	**0.017**
c. IFO-380 + C9500				
Concentration	9.132	**0.002**	1.303	0.333
Time	8.833	**0.007**	10.925	**0.003**
Concentration x Time	2.969	**0.009**	1.883	0.097
d. Slickgone				
Concentration	12.818	**0.001**	7.475	**0.005**
Time	19.971	**0.001**	21.680	**0.000**
Concentration x Time	3.273	**0.005**	2.847	**0.017**
e. C9500				
Concentration	22.531	**0.000**	13.643	**0.000**
Time	6.125	**0.019**	3.171	0.085
Concentration x Time	4.485	**0.001**	2.286	**0.046**

Table 4.13: One way analysis of variance (ANOVA) of the effective quantum yield of *H. ovalis* exposed to the IFO-380, IFO-380 + Slickgone, IFO-380 + Corexit 9500 (C9500), Slickgone alone and Corexit 9500 (C9500) alone treatments. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text. nc denotes ANOVA not calculated (no significant difference in the RmANOVA- Table 4.3). Values in bold denote a significant difference at P = 0.05.

Treatment		Exposure					Recovery			
		2	4	6	8	10	24	48	72	96
IFO-380	*F*	4.100	12.328	3.554	0.769	2.028	nc	nc	nc	nc
	P	**0.032**	**0.001**	**0.047**	0.569	0.166	nc	nc	nc	nc
IFO-380 + Slickgone	*F*	2.131	7.371	13.895	15.224	3.066	17.950	2.910	2.640	5.273
	P	0.151	**0.005**	**0.000**	**0.000**	0.069	**0.000**	0.078	0.097	**0.015**
IFO-380 + C9500	*F*	4.468	5.149	4.354	3.491	5.955	4.963	1.788	0.422	1.614
	P	**0.025**	**0.016**	**0.027**	0.050	**0.010**	**0.018**	0.208	0.776	0.245
Slickgone	*F*	1.955	1.893	2.197	7.958	1.495	0.439	0.114	2.441	1.671
	P	0.178	0.188	0.143	**0.004**	0.276	0.778	0.975	0.115	0.232
C9500	*F*	49.489	9.556	13.420	5.960	14.615	7.778	3.842	17.763	9.691
	P	**0.000**	**0.002**	**0.000**	**0.010**	**0.000**	**0.004**	**0.038**	**0.000**	**0.002**

Table 4.14: One way analysis of variance (ANOVA) of chlorophyll *a* pigments in *H. ovalis* at ten and 96 hours exposed to IFO-380, IFO-380 + Slickgone, IFO-380 + C9500, Slickgone alone and C9500 alone. Values in bold denote significant differences at $p = 0.05$ ($n = 3$).

	Treatment	0.00%	0.20%	0.40%	1.00%	2.00%	*F*	*P*
10 h	IFO-380	16.8 ± 0.7	16.5 ± 0.9	16.5 ± 0.9	15.4 ± 0.5	14.8 ± 0.6	1.146	0.217
	IFO-380 + Slickgone	16.8 ± 0.7	15.0 ± 1.8	16.0 ± 0.7	16.7 ± 0.4	14.8 ± 1.0	0.783	0.451
	IFO-380 + C9500	14.8 ± 1.2	14.6 ± 1.1	12.2 ± 2.0	11.0 ± 2.3	10.5 ± 1.8	1.283	0.249
	Slickgone	16.5 ± 0.3	12.8 ± 3.1	13.2 ± 3.2	12.1 ± 2.7	14.2 ± 0.6	0.528	0.516
	C9500	15.9 ± 0.0	12.9 ± 1.0	15.2 ± 0.5	14.7 ± 1.6	16.1 ± 2.0	0.944	0.484
96 h	IFO-380	**16.2 ± 0.2^{a}**	**13.0 ± 0.8^{b}**	**12.5 ± 0.5^{b}**	**11.6 ± 0.6^{b}**	**11.4 ± 0.4^{b}**	**13.926**	**0.001**
	IFO-380 + Slickgone	17.0 ± 0.6	16.2 ± 1.3	15.7 ± 0.9	12.9 ± 2.1	15.4 ± 1.0	1.457	0.203
	IFO-380 + C9500	**16.2 ± 0.2^{a}**	**15.7 ± 0.5^{ab}**	**13.2 ± 2.2^{ab}**	**10.8 ± 2.0^{ab}**	**9.7 ± 0.9^{b}**	**4.362**	**0.031**
	Slickgone	12.5 ± 2.2	8.9 ± 2.3	11.4 ± 1.4	10.0 ± 1.6	8.4 ± 0.5	0.988	0.324
	C9500	**16.1 ± 0.2^{a}**	**12.6 ± 1.7^{ab}**	**12.1 ± 1.3^{ab}**	**8.7 ± 0.3^{b}**	**8.7 ± 0.3^{b}**	**10.347**	**0.001**

4.4 Discussion

4.4.1 Tapis crude oil: non-dispersed, dispersed and dispersant alone

The toxicity of crude oil is often determined by the duration of time between the spill and the contact with an organism (Dodd 1974). Many studies support this showing minimal, if any, impact to organisms from weathered crude oil. With increasing duration post spill, the oil has a longer weathering time resulting in a greater loss of the volatile and toxic components, and therefore less toxicity to the organism. The experiments in this study support this finding in that the crude alone treatment, weathered for 24 hours, did not negatively impact either *Z. capricorni* or *H. ovalis*. This was supported by both the assessment of the chlorophyll *a* fluorescence data ($\Delta F/F_m'$) and the chlorophyll pigment analyses for both species. This is in clear contrast to a similar study by Macinnis and Ralph (2003a) where they found *Z. capricorni* declined in the effective quantum yield in laboratory exposures to aged crude oil and remained depressed even following replenishment of fresh seawater. However, most studies do support the finding that crude oil exhibits minimal toxicity to seagrass including Hatcher and Larkum (1982) assessing *Posidonia australis*; Thorhaug *et al.* (1986) assessing three tropical seagrass species; and Ralph and Burchett (1998b) assessing *H. ovalis*. The concentrations of petrochemicals used in these experiments were high, greater than those used in the Macinnis and Ralph (2003a) study (1.00 % w/v) which showed a negative impact to *Z. capricorni*. GCMS analysis of the 1.00 % crude treatment in this study (Chapter Two; Section 2.3.3; Figure 2.1) showed the sample to contain approximately 12 mg L^{-1} TPH which is quite high for a water accommodated fraction of crude oil (Reddy & Quinn 1999; Singer *et al.* 2000). Although the 2.00 % WAF treatment was not assessed with GC-MS it is reasonable to suggest that it was greater than 12 mg L^{-1}. Considering that laboratory experiments often exaggerate the effects of oil and yet even the highest concentrations of the crude oil did not evoke a significant response from either species is an important finding for oil spill mitigation.

The concern with dispersing an oil slick and the associated increased hydrocarbon content in the water column is somewhat supported by the results of the dispersed crude treatments to *Z. capricorni* but less so to *H. ovalis*. The toxicity of the dispersed crude oil to *Z. capricorni* was specifically related to the actual dispersant used and the petrochemical loading in the water column. These results agree with previous research in that different combinations of petrochemicals impact differently on seagrass (eg. Thorhaug *et al.* 1986). In this study, whilst, both Corexit 9527 dispersed and Ardrox dispersed crude oil treatments produced negative impacts to *Z. capricorni*, the Ardrox dispersed treatment had a more sustained negative impact on the seagrass. Following replenishment with 'fresh' seawater, photosynthetic health of the seagrass was still clearly inhibited in the Ardrox dispersed treatment. This prolonged impact may suggest possible damage to the photosynthetic apparatus, rather than just suppression of the photosynthetic output.

The response of *H. ovalis* to the dispersed oil and dispersant alone treatments was clearly different to the response of *Z. capricorni*. *Halophila ovalis* has been shown to be one of the most sensitive species in a wide range of studies including ultraviolet elevation (Dawson & Dennison 1996), light inhibition (Cheshire *et al.* 2002) and pollutants (Haynes *et al.* 2000). The thickness of the blade of *H. ovalis* is less than that in other seagrass (Enriquez *et al.* 2002) and it was considered that the petrochemicals may penetrate and disrupt the photosynthetic apparatus more rapidly than in *Z. capricorni*. In this study, the $\Delta F/F_m'$ of *H. ovalis* was impacted only within the first four hours when exposed to either of the dispersed crude treatments which was clearly different to the response of *Z. capricorni*. Although, the crude dispersed with Ardrox treatment led to an increase in the chlorophyll *a* pigment concentrations above that of the control of *H. ovalis* this only occurred at the ten hour sampling time and no differences were evident at 96 hours. These findings generally support the findings of Ralph and Burchett (1998b) in that *H. ovalis* showed minimal impact from dispersed oil and dispersant alone treatments. Considering, however, that *H. ovalis* is commonly found within *Z. capricorni* meadows, any impact to *Z. capricorni* must also be considered as a cause for concern to *H. ovalis*. If the *Z. capricorni* meadow becomes

compromised then the provision of habitat the meadow provides for *H. ovalis* is also compromised.

Interestingly, the total petroleum hydrocarbon (TPH) concentration remaining correlated with the impacts detected in the effective quantum yield when the seagrass were exposed to the dispersed crude treatments. The greater loss of TPH in the Ardrox dispersed treatment than in the Corexit 9527 treatment, correlated with the greater impact to the seagrass from the Ardrox dispersed treatment. In the Corexit 9527 dispersed crude treatment, only minimal loss of TPH occurred and less photosynthetic impact to the seagrass occurred. It is likely from this finding that the seagrass is actually either taking up the petrochemicals or the petrochemicals are adhering to the seagrass blade.

Unlike most of the impacts from the dispersed oil treatments, the dispersant alone treatments resulted in significant negative impacts to the seagrass even from the lower concentrations (0.20 and 0.40 % WAF). "Both *Z. capricorni* and *H. ovalis* were significantly impacted with exposure to the Ardrox alone treatment. *Z. capricorni* was also significantly impacted by the Corexit 9527 alone treatment. In the case of Corexit 9527 exposure to Z. *capricorni*, the impact was far greater than the dispersant- oil combination. The results from this study suggest that care still needs to be taken not to over disperse a slick as in some cases the dispersant alone treatment was more toxic than the oil-dispersant combination as in the case of the Corexit 9527 and *Z. capricorni*.

4.4.2 IFO-380: non-dispersed, dispersed and dispersant alone

The toxicity of bunker oil to organisms is often thought to be due to the increased persistence of the oil in the environment (API 1999; NRC 2005). The minor response of the chlorophyll *a* fluorescence data ($\Delta F/F_m'$) and the absence of significant differences in the chlorophyll pigment analysis for *Z. capricorni* exposed to the IFO-380 WAF treatment correlated with these assumptions from previous studies. It was interesting, therefore, to find that *H. ovalis* was impacted by the IFO-380 alone WAF treatment within the first two hours of exposure. Although there were no effects detected after six

hours exposure, this immediate impact to *H. ovalis* was surprising and somewhat alarming. Furthermore, although somewhat delayed at 96 hours sampling time, the chlorophyll pigment data showed all IFO-380 treatments significantly lower in chlorophyll *a* pigment concentration compared with the control. The TPH of the 1.00 % WAF treatment was about 2800 $\mu g\ L^{-1}$ with 25 % of this comprising the highly volatile, C_6 to C_9, carbon chain length components. The significant loss of TPH over the exposure period was most likely the loss of these lower weight components (Dodd 1974; API 1999). However, regardless of loss of TPH, there was still an impact to *H. ovalis* yet it remains unclear whether this was due to the chemical toxicity of the oil or an effect on the seagrass from the physical properties of the oil, such as smothering.

The fact that the IFO-380 did result in an acute toxic impact to *H. ovalis* should warrant cause for concern in oil spill mitigation. It means that the species can be impacted by the oil alone even without dispersing the slick and the associated increase in TPH dispersants add to the water column. This finding is alarming in that it poses a major problem for oil spill mitigation, especially when the addition of a dispersant to a fuel oil spill further negatively impacts the seagrass. Where a response was detected in the dispersed IFO-380 treatments it was generally far greater than that which occurred from the non – dispersed IFO-380. This is in agreement with Burridge and Shir (1995) where dispersed bunker oil treatments showed greater impact to coral nubbins than the bunker oil alone treatments. Hatcher and Larkum (1982) suggested that the decrease in light penetration from a dispersed crude oil played a role in the reduction in photosynthesis in *Posidonia australis*. The dispersed IFO-380 treatments in the current study, greatly altered the clarity of the solution in the treatment tanks with the water accommodated fraction resembling a rusty colour. Similarly to Hatcher and Larkum (1982), it is likely that these treatments reduced the amount of light penetrating to the seagrass within the tanks and may have aided in the decrease in effective quantum yield.

The effects of the two dispersed IFO-380 treatments varied between treatments and between species. *Zostera capricorni* was impacted only by the Corexit 9500 dispersed IFO-380 treatment whereas *H. ovalis* was impacted by both dispersed IFO-380 treatments. There were significant differences detected in the chlorophyll *a* pigment

analyses for *H. ovalis* exposed to the IFO-380 dispersed with Corexit 9500 treatment, but no differences detected in *Z. capricorni* exposed to the same treatment. There were also differences in the timing of the response of the seagrass. The effects to *Z. capricorni* from the Corexit 9500 treatment most notably followed the removal of the chambers, whereas to *H. ovalis* this effect was immediate. The severe impact to *H. ovalis* from the Slickgone dispersed IFO-380 over the exposure period was clearly different from the response of *Z. capricorni* which was not impacted negatively by the treatment.

The dispersant alone treatments also resulted in differences between treatments within the one species and differences between species response from the same treatment. *Halophila ovalis* was again impacted more severely than *Z. capricorni* when exposed to the same dispersant treatment. The Corexit 9500 alone treatment resulted in the greatest negative impact compared with any of the treatments to either of the species with the lack of recovery clearly cause for concern. The Slickgone alone treatment had the least impact on either species, but the severe impact by the Slickgone dispersed IFO-380 to *H. ovalis* complicates these findings. However, where a *Z. capricorni* meadow exists independently to *H. ovalis* then this research suggests dispersing the slick with Slickgone is more favourable than Corexit 9500.

It is clear that different petrochemical combinations impact differently within the same species and impacts between species can differ with the same petrochemical combination. The two species were tested simultaneously in the one tank to reduce the time required to run such experiments and to reduce differences between WAF between experiments. As such, both species were exposed to the same water accommodated fraction of each treatment and under the same conditions. This clearly shows the difference in the response between species is simply that, a difference in response between species and not an experimental artefact. This is a concern for oil spill mitigation when both species are present in the one environment and is one of the major problems of what to do in an oil spill. All science can do is provide information and the oil spill manager must make the decision based on these and other findings.

4.5 General Conclusions

In summary, from these experiments, the non-dispersed crude produced less damage to both *Z. capricorni* and *H. ovalis* than the dispersed oil combinations. Impacts to both species from the dispersed crude treatments were only detectable during the exposure day apart from the Ardrox dispersed crude treatment which continued to show impacts during the recovery days. The dispersant alone treatments showed impacts to both species but the response from the Ardrox alone treatment was greater than the Corexit 9527 alone treatment for both species.

The IFO-380 dispersed with Corexit 9500 had a far greater impact on both species than the IFO-380 alone. The IFO-380 and Slickgone resulted in no effect to *Z. capricorni*, but clearly inhibited *H. ovalis* up to the first recovery day. The Corexit 9500 alone treatment severely impacted *H. ovalis* and less so to *Z. capricorni*. The Slickgone alone treatment impacted *H. ovalis* more severely than *Z. capricorni* which showed no response over the exposure or recovery days.

The decision making process for mitigation managers is made more difficult when two species in one area show different responses to petrochemicals. Whilst *H. ovalis* is commonly patchily distributed it is also commonly found within the same area as *Z. capricorni*. Although one species may be less severely impacted by a particular petrochemical, any impact to either *H. ovalis* or *Z. capricorni* needs to be considered in mitigation procedures when they are present in the same environment. With prudence, it may be more beneficial however, for a negative response from *Z. capricorni* to be considered primarily above the response of *H. ovalis*. This is because any loss to the *Z. capricorni* meadow is likely to have a flow on negative effect to the *H. ovalis* meadow regardless of any lack of petrochemical impact to *H. ovalis*. Furthermore, *H. ovalis* is an opportunistic coloniser and may recover faster than larger slower growing species (Cheshire *et al.* 2002; Bryars & Neverauskas 2004), such as *Z. capricorni*.

The chlorophyll *a* fluorescence technique detected more rapid and comprehensive impacts on the seagrass than the analyses of the chlorophyll *a* pigment concentration.

Where differences were detected in the pigment analyses these were mainly detected at 96 hours following the initial petrochemical exposure; mainly within the IFO-380 treatments (dispersed, non-dispersed and dispersant alone); and almost solely detected in *H. ovalis*. It was interesting to find that the chlorophyll *a* pigment concentration of *H. ovalis* was clearly impacted by some of the petrochemical treatments, whereas only one petrochemical treatment (the crude dispersed with Ardrox) led to a significant impact to *Z. capricorni*. Further, this impact to *Z. capricorni* was an actual increase in chlorophyll *a* concentration due to the petrochemical treatment rather than a decrease. Ralph (1998) found the chlorophyll *a* pigment concentration to be a beneficial detector of osmotic stress to *H. ovalis* within a five hour period whereas Macinnis and Ralph (2003a) detected no differences in the chlorophyll *a* pigment concentration of *Z. capricorni* in an assessment of heavy metals exposure *in situ* but did detect differences in the chlorophyll *a*/*b* ratio and in the carotene concentration. A more comprehensive analysis of these pigments may have been useful in this study in assessing the health of *Z. capricorni* and is recommended for future studies.

These experiments generally show little impact from the treatments below the 1.00 % and 2.00 % WSF treatments. Most concentrations below this value elicited limited negative variation in the effective quantum yield. These results suggest that at low concentrations the seagrasses, both *Z. capricorni* and *H. ovalis*, are generally not negatively impacted in terms of photosynthetic stress. For mitigation managers this is highly useful information in that the focus of to disperse or not disperse may be placed on other resources surrounding an oil spill, with greater certainty as to the impacts to the seagrass. For those spills which equate to a 1.00 % WAF treatment or greater, careful consideration needs to apply regarding the decision to disperse or not to disperse.

Oil-in-water fluorometers are not designed for high-level petrochemical analysis. They are designed for a rapid determination of the general hydrocarbon content and the wavelength range they use to detect this is quite broad (Lambert e*t al.* 2003; Kim *et al.* 2010). The use of oil -in-water fluorometers was useful in this study as it provided a measure of loss of total petroleum hydrocarbon (TPH) concentration over the exposure period. It is clear from the TPH values that nowhere near all of the petrochemicals were

removed from the water body at the conclusion of the exposure period. Despite this, some (if not most) samples of seagrass still showed recoveryin the preceeding period. The seagrass actually recovers while the hydrocarbons are still in the surrounding water. It suggests that a negative impact to the seagrass may simply be inhibitory and not necessarily lead to mortality. Knowledge of the chemical composition of the petrochemicals throughout the experiment, however, would be useful. This would further assess whether the more toxic components remain in the water column and is one of the downfalls of current oil-in-water fluorometers.

Some dispersant alone treatments showed greater effect during the recovery days when compared with the results during the exposure day, eg. the increased stress response detected in the recovery days for *Z. capricorni* from the Corexit 9500 alone treatment (Figure 4.18). Macinnis and Ralph (2003a) found similar findings with dispersant exposure to *Z. capricorni* in laboratory experiments. The dispersant VDC led to no impact during the ten hour exposure period in their study, but did negatively impact the seagrass following the flushing of the exposure tanks and the consequent recovery measurements (Macinnis & Ralph 2003a). These findings suggest a delayed photosynthetic stress response from the seagrass. In this study, the dispersant alone treatments resulted in a uniform milky solution throughout the tank that was clearly in contact with the seagrass blade. At the conclusion of the exposure period the treatments tanks were drained, cleaned and refilled with fresh seawater. It is possible, however, that at least some of the chemical remained on the seagrass even after replenishment with the fresh seawater and could explain this increased impact with increased time. However, considering it was only the Corexit 9500 dispersant in this study which led to a delayed response it is likely the dispersant itself was more toxic to the seagrass.

Tank experiments cannot replicate microbial action as in the field (Clark and Noles 1994). Seagrasses were maintained in these experiments in approximately 3 cm of sediment taken from the area they were collected in. However, this cannot replicate the microbial interactions within the water column that would have occurred post exposure. Field experiments enabled a replenishment of the surrounding water, including the microbes within the water column, following the exposure period. In the tank

experiments the replenishing water was simply filtered seawater. If microbial action plays a role in the reduction of petrochemicals in the water column post exposure then the tank experiments are almost exclusively devoid of this element. This aspect was beyond the scope of this study but would be extremely useful to investigate in future experiments.

Laboratory experiments require less logistical time, effort and cost than *in situ* field experiments. These whole plant exposure experiments give evidence of this. More treatments (more oil and dispersant combinations), greater concentrations within treatments and an extra species, morphologically different to *Z. capricorni*, could be analysed in these laboratory experiments than in the field component. These laboratory experiments, however, still required a great deal of time to prepare for and to actually conduct. Several days were required to simply weather the oils, whilst the experiments themselves ran for 96 hours. A more rapid determination of seagrass response to petrochemicals would be beneficial to enable further scientific knowledge to be derived. Further, a testing protocol that could be used post spill to assist with the determination of the exact spill response would enable better mitigation decisions to be made.

5 Development of a Laboratory Testing Protocol

5.1 Introduction

"Window of opportunity" is the term often used in oil spill mitigation procedures denoting the short time frame that a decision "to disperse" or "not to disperse" is to be made. This "window of opportunity" can range from as little as several hours to one or two days post spill (NRC 2005). In Australia this timeframe may be reduced by an increased logistical difficulty associated with the expanse of the Australian coastline (Lipscombe 2000). The decision whether to disperse or not is highly complex. It is dependent upon the environment into which the oil has been spilt, abiotic and biotic factors within that area, and oil type and volume, amongst other factors. During this "window of opportunity", an assessment of all available information regarding the above factors is undertaken by the oil spill mitigation managers (NRC 2005). Even where comprehensive scientific data are available on an area, the organisms within and the oils themselves, a decision about what is likely to cause the least impact to the environment is still difficult (NRC 2005). More often than not, scientific data are lacking as there is simply no possibility to study every environmental resource under every possible oil spill scenario. In other circumstances, the scientific data may be contradictive. This is the case with subtidal seagrass (Macinnis & Ralph 2003a). Previous studies addressing the effects of dispersed and non-dispersed oil on subtidal seagrass showing somewhat conflicting results provides little assistance in determining the optimal mitigation strategy.

Research into the effects of oil spills is constrained by the logistical challenges of field experiments, whereas laboratory experiments are criticised for being unrealistic (NRC 2005). Field experiments provide the best representation of an organism's response when an oil spill occurs, but are costly in terms of time, effort and finances (Clark & Noles 1994). If a laboratory testing protocol for petrochemical impact to seagrass meadows was developed, then not only would more comparable tests be conducted,

furthering our knowledge *prior* to oil spills occurring, but tests could be also be carried out as soon as a spill occurs to determine the best mode of mitigation.

The short time frame of the "window of opportunity" denotes that testing methods need to be rapid. The testing methods also need to be simple to set up and to run. Common methods to assess the health of seagrass include Pulse Amplitude Modulated fluorometry (PAM) and analysis of the photosynthetic pigment concentrations. Both of these methods have been widely used in seagrass research and have been shown to be effective in detecting photosynthetic stress to seagrass (Ralph 1999; Haynes *et al.* 2000; Chesworth *et al.* 2004). Whilst fluorometry has been shown to be a sensitive and rapid indicator of photosynthetic stress, photosynthetic pigment analysis appears to be less sensitive in detecting stress; but may still provide supportive information in the determination of impacts (Macinnis & Ralph 2003a, b). A rapid detection of the semi-quantitative concentration of oil in the water column by oil-in-water fluorometers would enable an indication of what is occurring in terms of loss of oil in the test system and may also be useful in a rapid laboratory testing protocol. While oil-in-water fluorometers cannot differentiate between fractions they may be beneficial as a simple and rapid analysis tool for total petroleum hydrocarbon concentration in the water column (Henry & Roberts 2001; Lambert 2003; Lambert *et al.* 2003; Kim *et al.* 2010).

The following experiments were conducted as a first step towards developing a simple and rapid laboratory testing protocol for oil spill analysis. This stage of the development of the protocol was specifically addressing the determination of the likely effects of non-dispersed oil and dispersed oil on *Z. capricorni*. The major aims of this chapter were to determine the effects of non-dispersed oil and dispersed oil on leafblades of *Z. capricorni* in a laboratory experiment. The specific objectives were to 1) determine the effect of petrochemical treatments at different concentrations; 2) determine the nature of response in relation to the level of impact, time of impact and recovery and 3) determine the effectiveness of a semi-quantitative measure of hydrocarbon concentration at detecting these impacts.

5.2 Methods

Forty mm sections of *Z. capricorni* leaf blade were cut from the second leaf blade of the plant, about 30 mm above the apical meristem. The cut surface was sealed with Vaseline to prevent petrochemical uptake directly through the open wound. Leaf blades were secured to the underside of the lid from a 70 mL plastic sample jar. Seawater containing oil and dispersants were added to the sample jar and then inverted to allow light to penetrate through to the seagrass blade. Experiments were conducted in an oscillating water bath (150 rpm, 22 °C) under irradiance of 150 μmol photon $m^{-2}s^{-1}$.

Effective quantum yield ($\Delta F/F_m'$) was measured using a Mini PAM (Walz, Germany) with a 2mm fiberoptic (PolyOptic, Australia) inserted through a hole in the base of the sample jar. Measurements were taken every ten minutes for one hour, and then half hourly for the following four hours.

Chlorophyll *a* pigment concentrations and the percentage total petroleum hydrocarbon (TPH) concentration remaining at the conclusion of the experiment were determined as in Chapter Four. For the determination of the chlorophyll *a* pigment concentrations, the entire leafblade section was used in this range of experiments, and there was only one sampling time, at the conclusion of the five-hour exposure period.

Petrochemical treatments consisted of Tapis crude oil alone, Tapis crude oil and Corexit 9527 ©, Tapis crude oil and Ardrox, Corexit 9527 alone, Ardrox alone, 380cSt fuel oil alone, 380cSt fuel oil and Slickgone, 380cSt fuel oil with Corexit 9500, Slickgone alone and Corexit 9500 alone. For each experiment, six concentrations of the specific petrochemical were used (0.00, 0.10, 0.20, 0.40, 1.00, 2.00 % w/v) each with three replicates.

5.3 Results

5.3.1 Tapis crude oil: non-dispersed, dispersed and dispersant alone

Petrochemical Analysis

Figure 5.1 displays the percentage total petroleum hydrocarbon (TPH) concentration remaining of the water accommodated fraction (WAF) treatments following five hours exposure. There was minimal loss of the crude alone WAF treatments over the five hour experiment (Figure 5.1) and no significant differences occurred in any concentration between the pre- and post – exposure measurements (Table 5.1). All concentrations in the crude dispersed with Corexit© 9527 WAF treatment decreased significantly over the exposure period (Figure 5.1; Table 5.1). The 2.00 % concentration displayed the greatest loss over the exposure period decreasing to 63 % of the pre–exposure concentration. Similarly, most concentrations in the crude dispersed with Ardrox WAF treatment decreased significantly over the exposure period (Figure 5.1; Table 5.1). Only the 0.10 % concentration displayed no significant loss over the exposure period.

For the dispersant alone treatments, the Corexit 9527 WAF treatment (Figure 5.1) showed no significant loss over the exposure period, whereas the Ardrox WAF treatment (Figure 5.1) displayed significant declines in the TPH in the 0.20 % and 2.00 % concentrations (Table 5.1).

Chlorophyll *a* fluorescence

The crude alone treatment led to significant effects to the $\Delta F/F_m'$ of *Zostera capricorni* leafblade (Figure 5.2) within the first hour of exposure and continuing throughout the following four hours of exposure (Table 5.2). The $\Delta F/F_m'$ of the seagrass exposed to the 2.00 % concentration was significantly lower than the control after twenty minutes exposure continuing through until 180 minutes exposure. The 1.00 % concentration also

led to a significant decrease in $\Delta F/F_m'$ below the control, but this only occurred within the first hour, at 20 and 30 minutes exposure. Most noteworthy in the crude alone exposures was the elevation in $\Delta F/F_m'$ of the lower concentrations, mostly the 0.10 %, but also the 0.20 % and to a lesser degree the 0.40 % concentration. The 0.10 % concentration was significantly elevated above the 1.00 and 2.00 % at all sampling times and was also significantly greater than the control at the conclusion of the experiment, 300 mins. Similarly, the 0.20 % was elevated above the 2.00 % from twenty minutes exposure onwards and different to the 1.00 % at most sampling times. This treatment, the 0.20 % concentration was also significantly elevated above the control at the conclusion of the experiment (300 minutes exposure).

The crude dispersed with Corexit 9527 WAF treatment (Figure 5.3) resulted in fluctuations in the $\Delta F/F_m'$ of the seagrass during the first hour and although there was a significant concentration effect (Table 5.2) there was no clear pattern of effect between concentrations. The remaining four hours exposure led to highly significant interaction effects in the $\Delta F/F_m'$ of the seagrass (Table 5.2) with significant differences occurring at each sampling time (Table 5.3). After three hours exposure, the $\Delta F/F_m'$ of the seagrass in the 0.20, 0.40, 1.00 and 2.00 % concentrations were significantly lower than the control (Table 5.2, Table 5.3). These concentrations remained lower than the control at five hours with the 2.00 % concentration significantly lower than all other treatments. In the last two hours of the experiment the 2.00 % concentration was more than 0.23 and 0.35 units less than that of the control. In the last three hours of the experiment, the 0.20, 0.40 and 1.00 % concentrations were at least 0.1 units below the control.

There were significant interaction effects during the first hour and the following four hours of exposure of the crude dispersed with Ardrox treatments (Figure 5.4, Table 5.2, Table 5.3). The $\Delta F/F_m'$ of the seagrass was quite variable during the first hour, but showed a more consistent pattern of effect in the subsequent four hours. The 1.00 and 2.00 % concentrations were significantly lower than the control at 40 and 60 minutes exposure and also over the final three hours of the experiment (Figure 5.4, Table 5.3). The $\Delta F/F_m'$ of the seagrass exposed to the 2.00 % concentration was at least 0.1 units below the control from about 40 minute's exposure until the conclusion of the

experiment. Interestingly, over the last two hours of exposure, even the 0.40 % treatment showed significant decreases below that of the control. Elevation of the $\Delta F/F_m$' of the seagrass exposed to the 0.10 % treatment led to significant differences to those of the 2.00 % concentration. These significant differences occurred after 40 minutes exposure and remained so until the conclusion of the experiment (Table 5.3).

The Corexit 9527 alone WAF treatment led to much variability in the $\Delta F/F_m'$ of *Z. capricorni* during the first hour, largely in the 1.00 and 2.00 % treatments, and resulted in a significant interaction effect (Figure 5.5,Table 5.2). There were significant main effects over the following four hours (Table 5.2) and the 2.00 % concentration was decreased in $\Delta F/F_m'$ up to the conclusion of the experiment (Figure 5.5). Although no treatments differed significantly at 120 minutes exposure, the 1.00 and 2.00 % treatments were significantly lower than the control for the last three hours of the experiment (Table 5.3). The 0.10 and 0.20 % concentrations were different to the 2.00 % over the last two hours of exposure, whilst the 0.40 % concentration was significantly different to the 2.00 % at the last sampling time (300 minutes).

The Ardrox alone WAF treatment led to a significant interaction effect to *Z. capricorni* within the first hour of exposure and significant main effects of time and concentration during the following four hours of exposure (Figure 5.6; Table 5.3). The $\Delta F/F_m'$ of the seagrass exposed to the 2.00 % concentration in the Ardrox alone WAF treatment was significantly less than the control during the first hour of exposure (Figure 5.6; Table 5.3). By 40 minutes exposure, the 2.00 % concentration decreased to 0.1 units below the control and did not recover above this value for the remainder of the experiment. No other concentration was different to the control during the first hour. Over the next four hours of exposure the two largest concentrations, 1.00 and 2.00 %, were significantly lower than the control. By the conclusion of the experiment, the 2.00 % concentration was significantly different to all treatments except the 1.00 %, whilst the 1.00 % concentration was also significantly different to the two lowest concentrations, 0.10 and 0.20 %. (Table 5.2, Table 5.3).

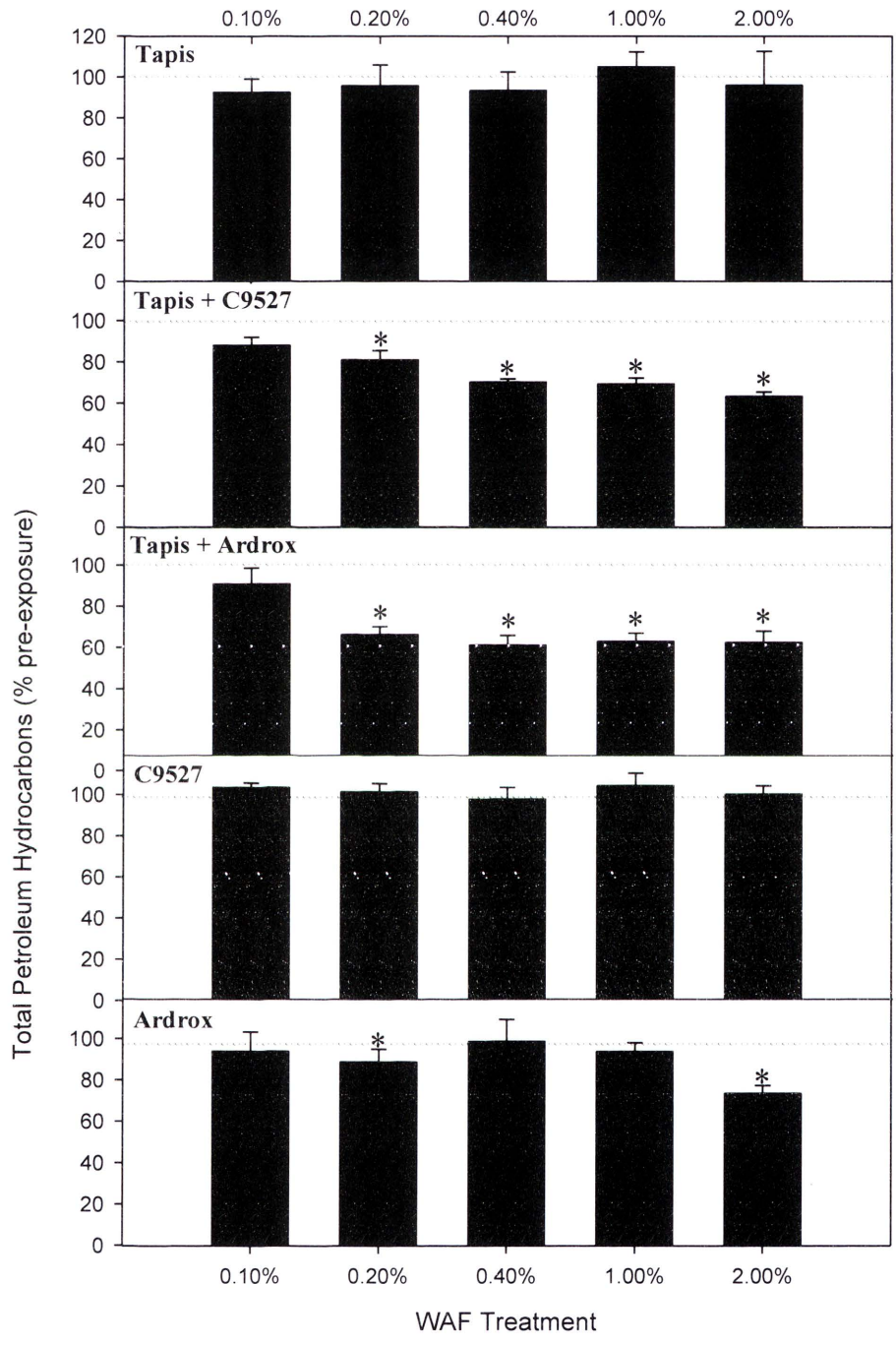

Figure 5.1: Percent TPH remaining of the water accommodated fraction following five hours laboratory exposure of Tapis crude oil alone, Tapis crude oil + Corexit 9527, Tapis crude oil + Ardrox, Corexit 9527 alone and Ardrox alone. Averages ± standard error of the mean are shown (n = 3).

Table 5.1: Independent *t* test analysis of the total hydrocarbon concentration pre– and post–exposure of Tapis crude oil alone, Tapis crude oil + C9527, Tapis crude oil + Ardrox, C9527 alone and Ardrox alone WAF treatments. Values in bold denote significant differences at $P = 0.05$.

Treatment	0.10 %		0.20 %		0.40 %		1.00 %		2.00%	
	t	*P*	t	*p*	t	*p*	t	*p*	t	*P*
Crude	0.98	0.38	0.66	0.54	0.77	0.48	0.25	0.58	0.38	0.72
Crude + C9527	2.67	**0.06**	5.34	**0.01**	19.0	**0.00**	11.9	**0.01**	20.01	**0.00**
Crude + Ardrox	0.98	0.38	5.96	**0.00**	8.48	**0.01**	9.32	**0.00**	2.96	0.10
C9527	0.62	0.57	0.24	0.82	0.33	0.76	0.78	0.48	0.09	0.92
Ardrox	0.53	0.63	3.13	**0.00**	0.27	0.80	1.62	0.18	24.98	**0.00**

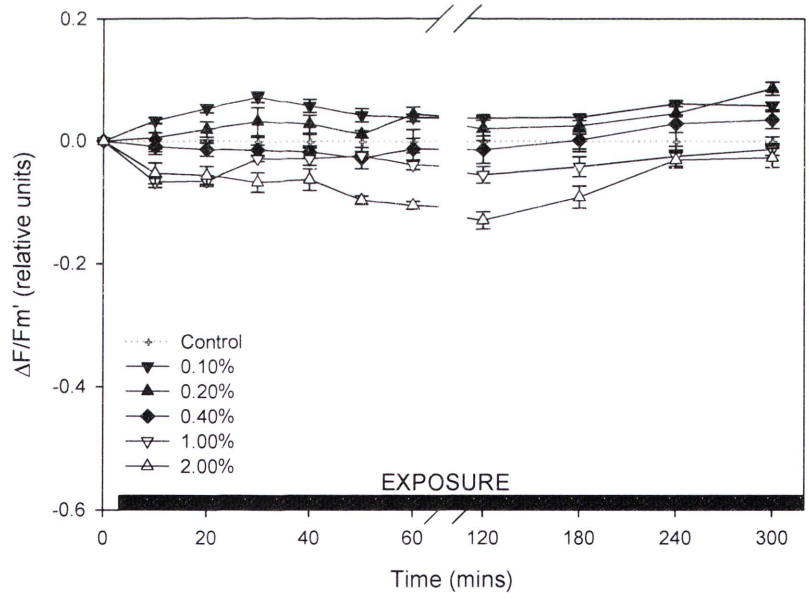

Figure 5.2: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of Tapis crude oil. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown ($n = 3$).

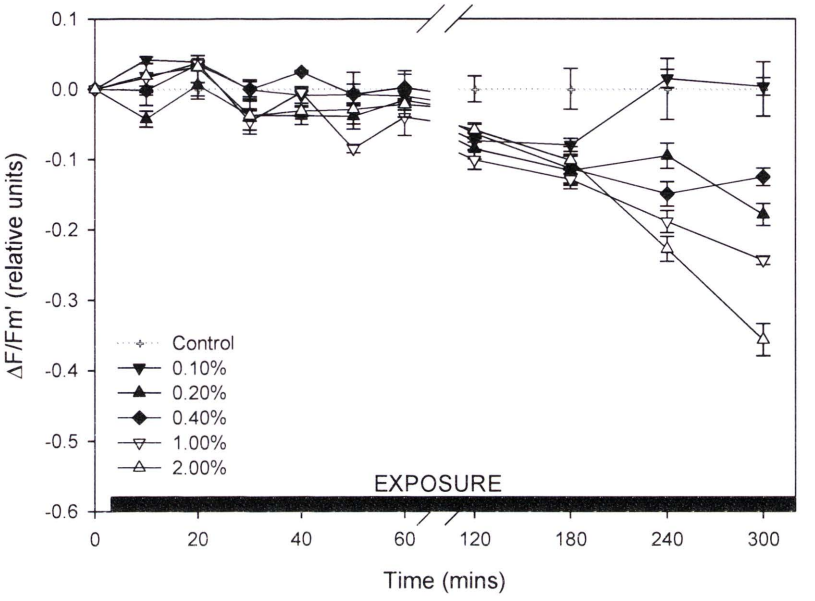

Figure 5.3: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of Tapis crude oil dispersed with Corexit 9527. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

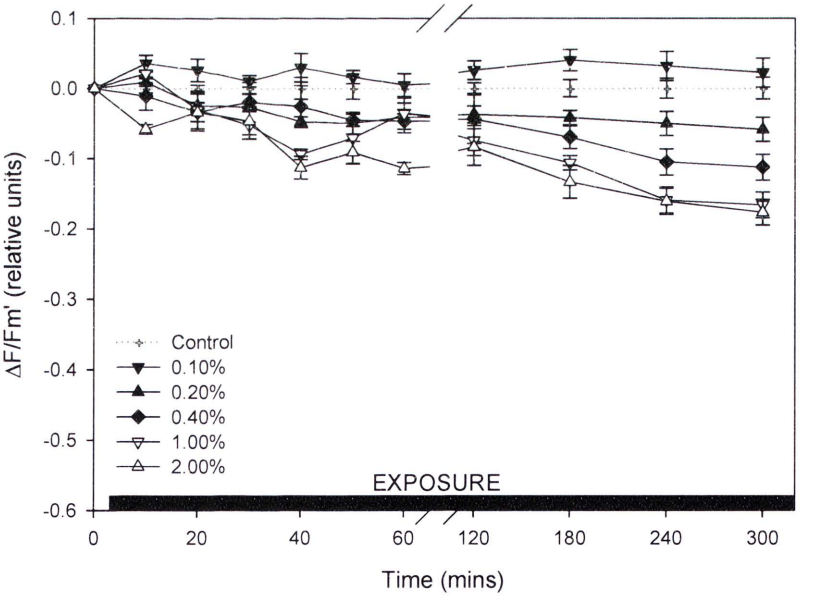

Figure 5.4: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of Tapis crude oil dispersed with Ardrox. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

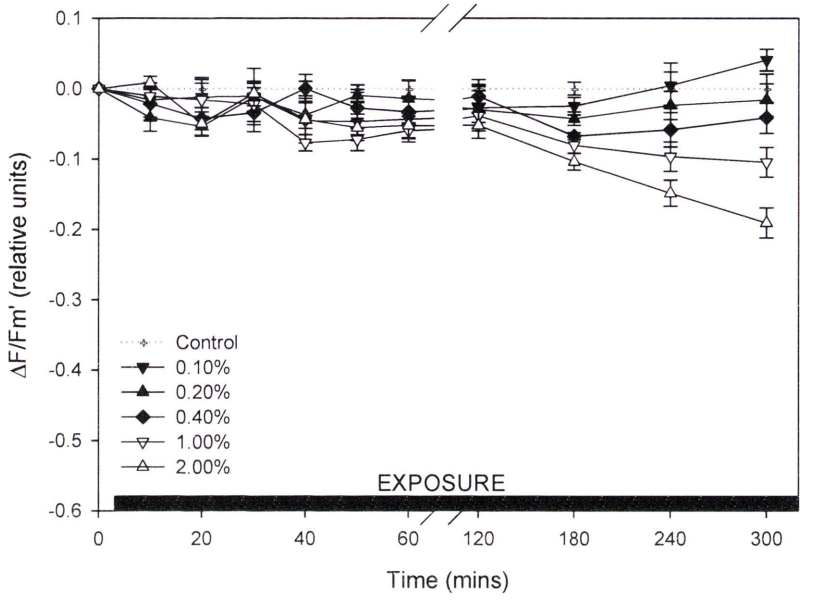

Figure 5.5: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of Corexit 9527. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n = 3).

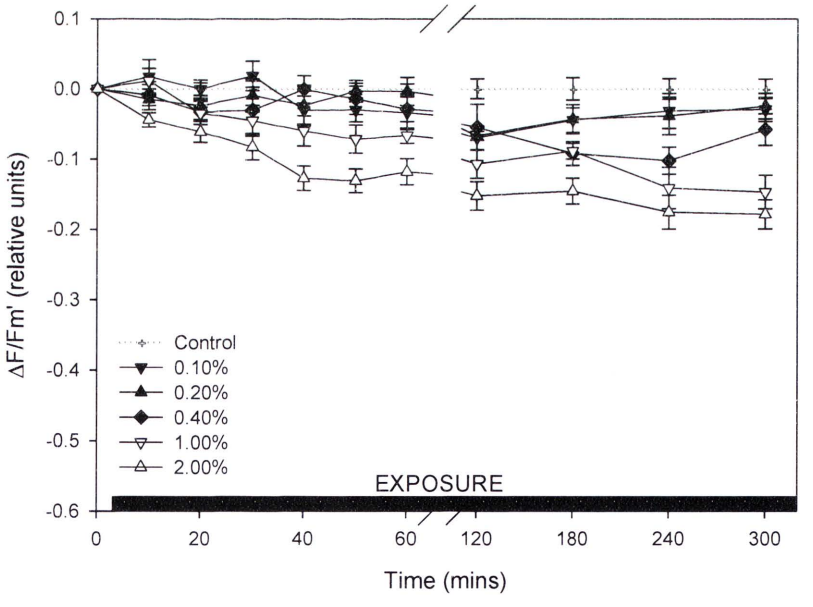

Figure 5.6: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of Ardrox. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

Photosynthetic pigment analysis

Chlorophyll *a* pigment analysis detected significant differences in the crude dispersed with Ardrox and the Corexit 9527 alone WAF treatments (Table 5.4). In the crude dispersed with Ardrox this was due to an increased chlorophyll *a* pigment concentration in the 0.10 % WAF treatment being different to a decrease in the 2.00 % WAF treatment. An elevation in the chlorophyll *a* pigment concentration was evident in the 0.10 % WAF treatment in the Corexit 9527 alone treatment compared with the control and other treatments (Table 5.4).

Table 5.2: Repeated measures ANOVA for the effective quantum yield data of *Z. capricorni* leafblades exposed to the different concentrations of a) Tapis crude oil alone, b) Tapis crude oil + C9527, c) Tapis crude oil + Ardrox, d) C9527 alone and e) Ardrox alone WAF treatments. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text. Values in bold denote significant difference at $P = 0.05$.

Treatment	Effect	Minutes		Hours	
		F	P	F	P
a. Tapis	Concentration	32.606	**0.000**	17.052	**0.000**
	Time	2.488	0.121	29.696	**0.000**
	Concentration x Time	1.470	0.113	2.426	**0.013**
b. Tapis + C9527	Concentration	3.682	**0.031**	16.798	**0.000**
	Time	3.483	0.055	33.262	**0.000**
	Concentration x Time	1.865	0.058	6.323	**0.000**
c. Tapis + Ardrox	Concentration	10.375	**0.000**	25.172	**0.000**
	Time	27.917	**0.000**	326.948	**0.000**
	Concentration x Time	2.280	**0.015**	7.849	**0.000**
d. C9527	Concentration	0.758	0.597	8.866	**0.001**
	Time	9.848	**0.003**	4.756	**0.024**
	Concentration x Time	2.614	**0.006**	1.847	0.061
e. Ardrox	Concentration	5.720	**0.006**	22.058	**0.000**
	Time	9.324	**0.003**	6.557	**0.009**
	Concentration x Time	1.904	**0.044**	1.161	0.347

Table 5.3: One-way ANOVA of the effective quantum yield data of *Z. capricorni* exposed to the different concentrations of Tapis crude oil, Tapis crude oil + C9527, Tapis crude oil + Ardrox, Corexit 9527 alone and Ardrox alone WAF treatments. Values in bold denote a significant difference at $P = 0.05$.

Treatment		Time									
		10	20	30	40	50	60	120	180	240	300
Tapis	*F*	6.90	19.73	14.49	11.19	16.53	18.90	14.94	10.66	7.54	15.59
	P	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Tapis + C9527	*F*	4.08	2.85	2.32	8.32	3.72	0.64	6.32	12.42	18.33	14.12
	P	**0.02**	**0.06**	0.11	**0.00**	**0.03**	0.68	**0.00**	**0.01**	**0.00**	**0.00**
Tapis + Ardrox	*F*	9.53	1.84	3.62	9.30	4.54	6.94	3.97	18.20	20.05	21.40
	P	**0.00**	0.18	**0.03**	**0.00**	**0.02**	**0.00**	**0.02**	**0.00**	**0.00**	**0.00**
C9527	*F*	1.03	1.30	0.24	2.58	5.10	1.22	1.63	7.80	6.43	16.11
	P	0.44	0.31	0.94	**0.08**	**0.03**	0.36	0.58	**0.00**	**0.00**	**0.00**
Ardrox	*F*	1.88	3.54	2.60	5.80	9.06	6.62	6.52	8.88	8.52	13.64
	P	0.17	**0.03**	**0.08**	**0.01**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

Table 5.4: One way analysis of variance (ANOVA) of chlorophyll *a* pigments of *Z. capricorni* leafblade section exposed to Tapis crude oil, Tapis crude oil + C9527, Tapis crude oil + Ardrox, C9527 alone and Ardrox alone. Bold denotes significant difference ($P < 0.05$). Average ± standard error of the mean ($n = 3$).

Treatment	Control	0.10 %	0.20 %	0.40 %	1.00 %	2.00%	*F*	*p*
Tapis	12.0 ± 2.3	10.6 ± 2.5	14.1± 1.9	11.2 ± 2.7	12.3 ± 2.4	14.9 ± 1.4	0.560	0.729
Tapis + C9527	12.5 ± 1.8	12.4 ± 2.0	12.5 ± 1.2	11.4 ± 1.6	10.5 ± 1.1	11.5 ± 0.6	0.306	0.900
Tapis + Ardrox	**14.4 ±1.7^{ab}**	**15.9 ± 0.9^{a}**	**14.4 ± 0.4^{ab}**	**13.1 ± 0.6^{ab}**	**14.4 ± 0.7^{ab}**	**10.7 ± 0.8^{b}**	**4.459**	**0.034**
C9527	**9.7 ± 1.3^{a}**	**14.0 ± 1.6^{b}**	**13.2 ± 0.9^{a}**	**10.5 ± 0.8^{a}**	**11.6 ± 0.5^{a}**	**10.3 ± 0.4^{a}**	**3.350**	**0.039**
Ardrox	13.4 ± 1.5	14.0 ± 1.6	14.7 ± 0.8	10.8 ± 2.6	11.6 ± 1.4	11.0 ± 0.4	1.216	0.359

5.3.2 IFO-380 oil: non-dispersed, dispersed and dispersant alone

Petrochemical Analysis

The percent total petroleum hydrocarbon (TPH) concentration remaining in the water accommodated fraction (WAF) treatments following the five hour exposure period are displayed in Figure 5.7. In the IFO-380 alone WAF treatment (Figure 5.7), the 2.00 % concentration significantly decreased to 70 % of the pre–exposure TPH concentration (Table 5.5). No other concentration differed significantly in this WAF treatment. Similarly, there was little variation in the dispersant alone WAF treatments with only the 0.40 % Slickgone alone WAF treatment displaying a significant decrease in TPH ($p = 0.01$; Table 5.5).

In both dispersed IFO-380 treatments (Figure 5.7) there were highly significant losses of TPH over the exposure period. The IFO-380 dispersed with Corexit 9500 WAF treatment displayed a greater relative loss of TPH with each increasing WAF treatment (Figure 5.7). The 0.10 % concentration was the only concentration to not have a significant loss in TPH over the exposure period. The largest loss occurred in the 2.00 % concentration, where approximately 25 % of the initial pre–exposure TPH was remaining post exposure (Figure 5.7). The IFO-380 dispersed with Slickgone WAF treatments displayed the greatest loss in TPH over the exposure period, compared with all other WAF treatments. There was a highly significant loss of TPH in all concentrations over the exposure period (Table 5.5; $p < 0.001$). Only 23 % of the initial pre-exposure concentration remained at the conclusion of the experiment. All other concentrations had between 33 and 36 % TPH remaining at the conclusion of the experiment (Figure 5.7).

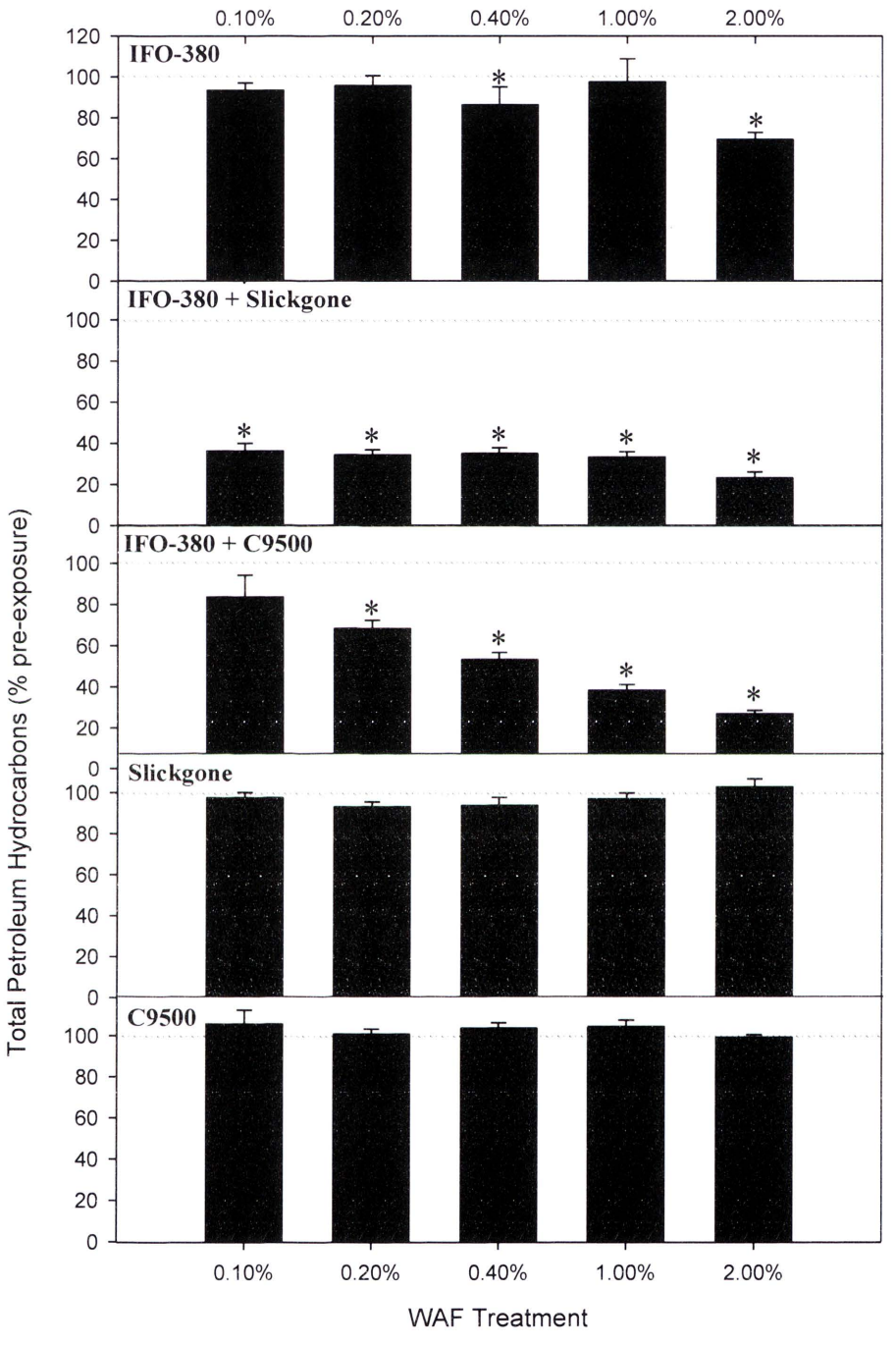

Figure 5.7: Percent TPH remaining of the water accommodated fraction following ten hours laboratory exposure of IFO-380 alone, IFO-380 + Slickgone, IFO-380 + Corexit 9500, Slickgone alone and Corexit 9500 alone. Average ± standard error of the mean are shown (n = 3).

Table 5.5: Independent *t* test analysis of the total hydrocarbon concentration pre– and post–exposure of IFO-380 alone, IFO-380 + Slickgone, IFO-380 + Corexit 9500, Slickgone alone and Corexit 9500 alone WAF treatments. Values in bold denote significant difference at $P = 0.05$.

Treatment	0.10 %		0.20 %		0.40 %		1.00 %		2.00%	
	t	*p*	t	*p*	t	*p*	t	*p*	T	*p*
IFO-380	0.8	0.49	1.1	0.31	2.1	**0.09**	0.2	0.84	10.8	**0.00**
IFO-380 + Slickgone	21.5	**0.00**	22.7	**0.00**	22.3	**0.00**	23.4	**0.00**	20.4	**0.00**
IFO-380 + C9500	1.6	0.19	8.2	**0.00**	15.4	**0.00**	70.8	**0.00**	45.2	**0.00**
Slickgone	1.7	0.16	3.9	0.15	4.5	0.10	1.3	0.27	0.5	0.66
C9500	0.9	0.42	0.4	0.74	2.0	0.12	1.8	0.14	0.24	0.82

Chlorophyll *a* fluorescence

Seagrasses exposed to the IFO-380 alone WAF treatment displayed minimal variation in the $\Delta F/F_m'$ of all WAF concentrations over the experimental period (Figure 5.8; Table 5.6,Table 5.7). The photosynthetic health of the seagrass exposed to the 2.00 % WAF concentration was significantly lower than the control, 0.10 and 0.20 % concentrations at 20 minutes exposure and lower than the control at 240 and 300 minutes (Table 5.6, Table 5.7). However, the $\Delta F/F_m'$ of the seagrass in this largest concentration was never more than 0.05 units (< 15 %) below that of the control at any sampling time.

There was much variability in the $\Delta F/F_m'$ of the seagrass exposed to the IFO-380 dispersed with Slickgone WAF treatment in the first hour (Figure 5.9). The only concentrations to differ from the control over the first hour were the 0.40 and 1.00 % concentrations (Figure 5.9). Over the following hours of the experiment both the 1.00 and 2.00 % concentrations were lower than the control (Figure 5.9) with both concentrations still significantly lower at the conclusion of the experiment (Table 5.6, Table 5.7). The 0.40 % concentration was also significantly lower than the control at two hours exposure. The 2.00 % concentration fell below 0.15 units from the $\Delta F/F_m'$ of the control after five hours exposure. Other than the 1.00 and 2.00 % concentrations, no

other concentrations differed significantly from the control over the experimental period (Table 5.6, Table 5.7).

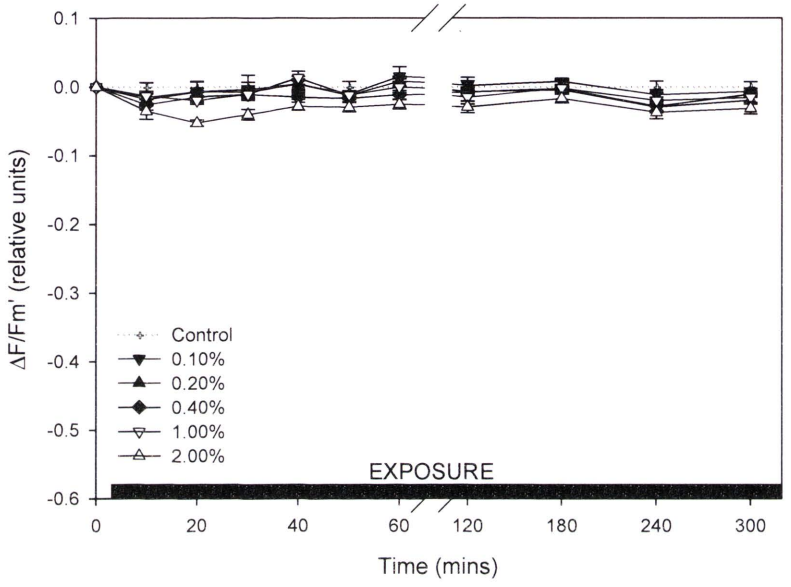

Figure 5.8: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of IFO-380. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

There were interaction effects in the $\Delta F/F_m'$ of the seagrass exposed to the IFO-380 dispersed with Corexit 9500 WAF treatment (Figure 5.10, Table 5.6). The different concentrations resulted in fluctuations in the $\Delta F/F_m'$ of *Z. capricorni* throughout the first hour of exposure followed by a decrease in the seagrass health over the next four hours of the experiment. The greatest depression in $\Delta F/F_m'$ in these experiments occurred with exposure to this treatment. The $\Delta F/F_m'$ of the seagrass in the 1.00 % concentration dropped over 0.2 units below the control and the 0.20 and 0.40 % dropped at least 0.1 units below the control (Figure 5.10). At the conclusion of the experiment, the 2.00 % concentration was significantly lower than all other concentrations including the control, decreasing to 0.23 and 0.35 units below the control at four and five hours respectively (Figure 5.10; Table 5.6). The 0.20, 0.40 and 1.00 % concentrations were also significantly lower than the control and the 0.10 % concentration at the conclusion of the experiment.

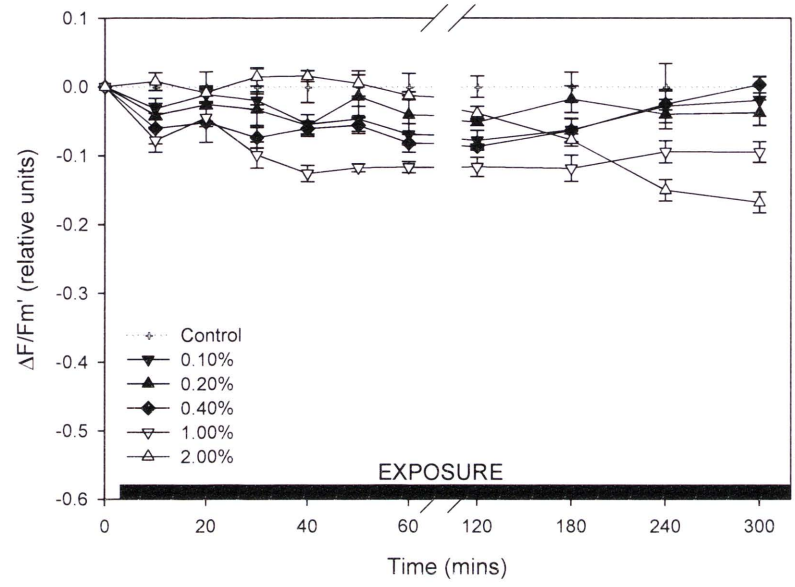

Figure 5.9: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of IFO-380 dispersed with Slickgone LTSW. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

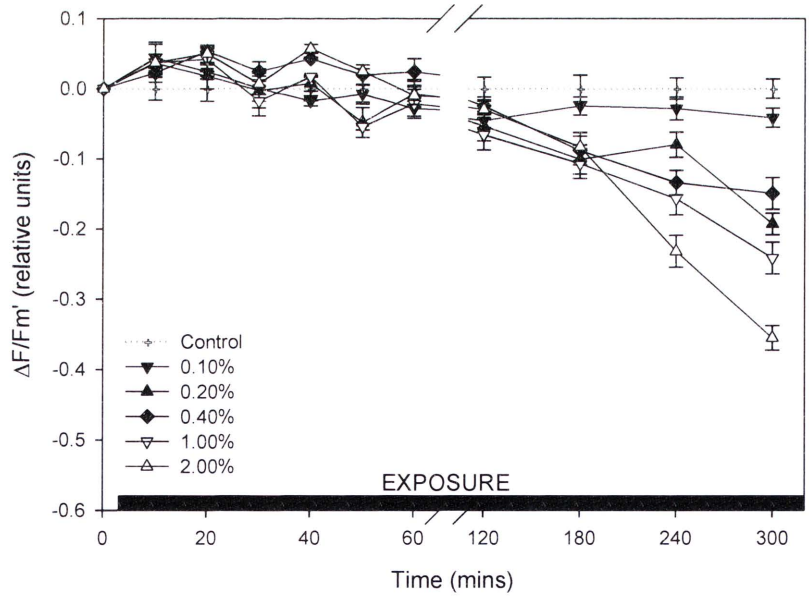

Figure 5.10: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of IFO-380 dispersed with Corexit 9500. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

During the first hour of exposure to the Slickgone alone WAF treatments there were no significant impacts to the photosynthetic health of the seagrass (Figure 5.11; Table 5.6, Table 5.7). The 1.00 and 2.00 % concentrations showed a negative response in the $\Delta F/F_m'$ of the seagrass only after four hours of exposure (Table 5.7) with the greatest reduction in $\Delta F/F_m'$, 0.11 units, in the 2.00 % concentration.

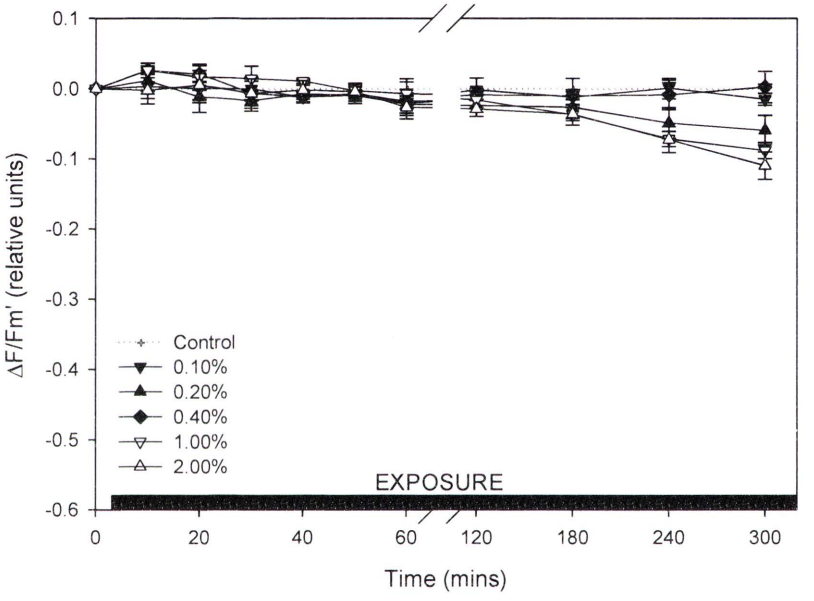

Figure 5.11: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of Slickgone. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

The seagrass exposed to the 1.00 and 2.00 % concentrations in the Corexit alone WAF treatment were negatively impacted within the first hour (Figure 5.12; Table 5.6, Table 5.7). There was some elevation in the $\Delta F/F_m'$ of the 0.10 % concentration leading to significant differences between this treatment and the 2.00 % concentration at most sampling times throughout the experiment (Table 5.7). The $\Delta F/F_m'$ of the 1.00 % and 2.00 % concentrations decreased up to the conclusion of the experiment at five hours (Figure 5.12). The 2.00 % differed to the control after 50 minutes exposure and remained significantly lower for the remainder of the experiment. The 1.00 % concentration differed to the control for the last three hours of measurements. The

$\Delta F/F_m'$ in both the 1.00 % and 2.00 % concentrations decreased to below 0.1 units below the control at four and five hour's exposure.

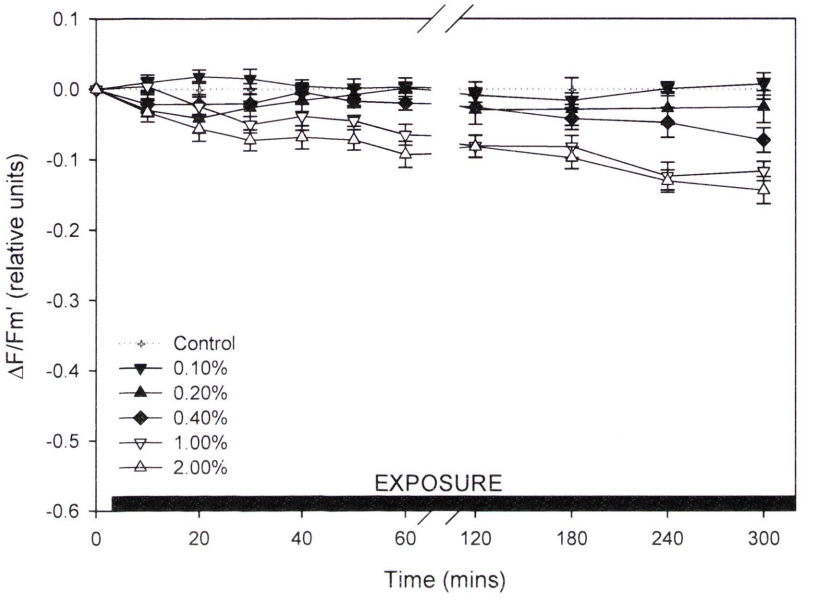

Figure 5.12: Change in effective quantum yield of *Z. capricorni* leafblade section exposed to the water accommodated fraction (WAF) of Corexit 9500. Time zero is pre-petrochemical exposure. Average ± standard error of the mean are shown (n =3).

Photosynthetic pigment analysis

Few significant differences were detected in the chlorophyll *a* pigment analyses (Table 5.8). The chlorophyll *a* pigment concentration of *Z. capricorni* exposed to the two largest Slickgone alone WAF treatments were significantly lower than that of the control and the lower WAF concentrations, 0.10, 0.20 and 0.40 % WAF ($p = 0.001$). In the Corexit 9500 alone treatments, the chlorophyll *a* pigment concentration exposed to the 2.00 % WAF concentration was significantly lower than that of the seagrass exposed to the 0.40 % WAF concentration ($p = 0.042$). No other treatments showed significant differences between the treatments and or control.

Table 5.6: Repeated measures ANOVA of the effective quantum yield data of *Z. capricorni* exposed to the different concentrations of a) IFO-380 alone, b) IFO-380 + Slickgone, c) IFO-380 + Corexit 9500 (C-9500), d) Slickgone alone and e) Corexit 9500 alone WAF treatments.

Treatment	Effect	Minutes		Hours	
		F	P	F	P
a. IFO-380	Concentration	4.536	**0.015**	3.267	**0.043**
	Time	6.087	**0.013**	18.691	**0.000**
	Concentration x Time	1.057	0.417	1.092	0.403
b. IFO-380 + Slickgone	Concentration	8.921	**0.001**	7.718	**0.003**
	Time	2.463	0.123	0.836	0.536
	Concentration x Time	1.044	0.450	3.608	**0.001**
c. IFO-380 + C9500	Concentration	2.103	0.135	20.307	**0.000**
	Time	15.247	**0.001**	84.892	**0.000**
	Concentration x Time	2.427	**0.010**	8.72	**0.000**
d. Slickgone	Concentration	0.461	0.798	9.851	**0.001**
	Time	3.106	0.075	3.974	**0.040**
	Concentration x Time	0.543	0.940	0.844	0.649
e. C9500	Concentration	6.204	0.005	13.671	**0.000**
	Time	1.634	0.256	3.242	0.066
	Concentration x Time	1.075	0.420	1.439	0.177

Table 5.7: One way ANOVA of the effective quantum yield data of *Z. capricorni* exposed to the different concentrations of IFO-380 alone, IFO-380 + Slickgone, IFO-380 + Corexit 9500 (C-9500), Slickgone alone and Corexit 9500 alone WAF treatments. Differences between concentrations were determined using Tukey's post hoc comparison and are described in the text.

Treatment		Time									
		10	20	30	40	50	60	120	180	240	300
IFO-380	*F*	2.14	4.90	1.83	3.49	2.65	2.32	1.68	2.07	3.26	2.79
	P	**0.13**	**0.01**	0.18	**0.04**	0.08	0.11	0.21	0.14	**0.04**	**0.07**
IFO-380 + Slickgone	*F*	4.43	1.16	5.38	12.13	5.98	7.12	12.74	5.83	6.03	16.01
	P	**0.02**	0.38	**0.01**	**0.00**	**0.00**	**0.00**	**0.00**	**0.01**	**0.00**	**0.00**
IFO-380 + C9500	*F*	0.85	2.13	0.52	8.60	5.22	1.287	2.08	5.16	20.62	53.02
	P	0.54	0.13	0.76	**0.00**	**0.01**	0.33	0.13	**0.01**	**0.00**	**0.00**
Slickgone	*F*	0.86	0.41	0.36	0.78	0.17	0.44	1.18	1.50	4.44	8.69
	P	0.54	0.83	0.87	0.59	0.97	0.81	0.37	0.26	**0.02**	**0.00**
C9500	*F*	1.87	3.27	6.08	3.15	6.56	10.18	4.36	5.60	11.84	12.14
	P	0.17	**0.04**	**0.00**	**0.03**	**0.00**	**0.00**	**0.02**	**0.01**	**0.00**	**0.00**

Table 5.8: One way analysis of variance (ANOVA) of chlorophyll *a* pigments in leafblades of *Z. capricorni* exposed to IFO-380 alone, IFO-380 + Corexit 9500 (C-9500), IFO-380 + Slickgone, Corexit 9500 alone and Slickgone alone. Bold denotes significant difference ($P < 0.05$). Averages ± standard error of the mean ($n = 3$).

Treatment	Control	0.10 %	0.20 %	0.40 %	1.00 %	2.00%	F	*P*
IFO-380	14.0±0.4	14.1±1.8	13.3±1.0	10.0±1.3	11.4±1.8	11.9±1.2	1.473	0.269
IFO-380 + Slickgone	11.5±2.6	13.1±1.7	12.1±1.8	14.1±1.5	15.2±0.6	11.2±3.0	0.603	0.699
IFO-380 + C9500	14.0±1.2	14.7±1.3	13.2±1.8	12.0±2.0	10.6±2.3	12.0±1.7	0.714	0.625
Slickgone	**14.9±0.8^{a}**	**13.0±0.6^{a}**	**14.3±0.8^{a}**	**11.2±1.3^{a}**	**10.1±0.3^{b}**	**8.8±0.7^{b}**	**8.470**	**0.001**
C9500	**10.6±0.4^{ab}**	**10.6±0.8^{ab}**	**10.5±1.3^{ab}**	**13.0±1.2^{a}**	**10.0±2.0b^{a}**	**6.9±0.7^{b}**	**2.348**	**0.042**

5.4 Discussion

Unlike in a real spill situation where sediment and other biota may act as a sink for the partitioning of petrochemicals, in these leafblade experiments there were only few sinks, the seagrass blade, the fibre optic and the internal walls of the exposure jars. There was no sediment present and the filtered seawater used in these experiments were likely to have contained minimal water-column bacteria. Real spill conditions, *in situ* experiments and even laboratory experiments where the sediment is included in the experiment would have much higher rates of biological activity than that which occurred within these leafblade experiments (Clark and Noles 1994). In these leafblade experiments, other than the seagrass blade itself, there would have been minimal biological activity to interact with the oil. As petrochemicals can be degraded by biological activity such as by microbial breakdown (Leahy & Colwell 1990; API 1999), it was considered that there would be minimal breakdown and loss of the petrochemicals within the sample jars. Whilst minimal loss was apparent in the oil alone and some of the dispersant alone treatments, the clear loss of total petroleum hydrocarbons (TPH) in the dispersed treatments was surprising.

All dispersed oil treatments exhibited a significant loss of TPH over the exposure period. As there were few compartmental sinks, the fate for these petrochemicals was likely to be in or on the seagrass blade itself, the fibre optic, or adherence to the internal wall of the sample jar (Clark & Noles 1994). As it was the dispersed oil treatments that also displayed the greatest photosynthetic impact to the seagrass, uptake or adherence to the seagrass blade in these treatments appears highly likely. Dispersants are thought to penetrate the waxy cuticle of the seagrass blade leading to a decreased tolerance of the seagrass to other stress factors (Zieman *et al.* 1984; Howard *et al.* 1989). Dispersants are also thought to accumulate in the chloroplasts chiefly within the thylakoid membrane (Howard *et al.* 1989; Wolfe *et al.* 1998). Interestingly, the dispersant alone treatments were less toxic to the seagrass, suggesting it was the combination of the oil and dispersant that led to the impacts.

There was minimal, if any, impact to the seagrass when exposed to the oil alone treatments. This coincided with minimal loss of TPH over the exposure period (Figure 5.1). GC-MS determination of the carbon-chain length fractionation (Figure 2.1, Chapter 2) showed that these treatments, especially the crude alone, had a much higher concentration of the highly toxic lower weight hydrocarbons than the dispersed oil treatments. These highly toxic components are lost rapidly in real spill situations (API 1999), but it was considered in these leafblade experiments that these components would lead to a negative photosynthetic effect to the seagrass. This was not apparent. There were some negative impacts to the crude alone treatment after 60 minutes exposure, but the seagrass recovered following this time. Similarly the most concentrated IFO-380 alone WAF treatment did display a significant difference from the control, but the decrease in $\Delta F/F_m'$ was far less than that seen in the dispersed oil treatments. The crude alone treatments displayed significant increases in $\Delta F/F_m'$ above the control. It is suggested that the oil alone was actually taken up by the leafblades and led to this stimulatory effect which is common throughout other oil spill research (eg. Karydis & Fogg 1980; Chan & Chiu 1985).

Many previous studies have shown negative photosynthetic and metabolic impacts to organisms from exposure to dispersant alone treatments (eg. Epstein *et al.* 2000; Shafir *et al.* 2000). The Corexit 9527 and Ardrox alone treatments clearly supported these findings even within the short, five hour exposure period of these experiments. The pre-exposure TPH of these two treatments was amongst the highest concentrations recorded from the GC-MS analysis (Figure 2.1, Chapter Two), up to 300 mg L^{-1} so it was reasonable to assume that the dispersants would negatively impact the seagrass. These treatments, Corexit 9527 and Ardrox, had the lowest concentration of the light weight components C_6-C_9, but comprised relatively high concentrations of the heavier carbon-chain fractions compared with the other treatments. Clark *et al.* (2001) suggested that medium weight molecular components are more toxic to marine life compared with the light weight components that are suggested as the most toxic by other authors (eg. API 1999). This greater toxicity was suggested due to the longer residence time of the medium weight components which are still considered acutely toxic to organisms in the environment compared with the rapid loss of the light weight components. Macinnis and Ralph (2003b) found that exposing *Z. capricorni* to the dispersant VDC led to

limited photosynthetic impacts during a ten-hour exposure period, but resulted in increased stress to the plant after replenishment of 'fresh' seawater in manipulated laboratory experiments. In this study, the Corexit 9500 and Slickgone treatments led to some minor reductions in $\Delta F/F_m'$ in these experiments, but it may be possible that greater impacts would eventuate had the experiment ran for a longer period of time.

Whilst there were clear and negative impacts from the dispersant alone treatments, especially the Corexit 9527 and Ardrox alone treatments, there was generally little correlation with the loss of TPH by the oil-in water fluorometer. The semi-quantitative method used to determine TPH in this experiment has its limitations and the anomaly in these dispersant alone results highlight these. This semi-quantitative measure cannot detect differences within the different components of the petrochemicals (Lambert *et al.* 2003). The Trilogy fluorometer measures TPH via a broad wavelength absorbance spectra and previous research suggests that this incorporates a large range of hydrocarbons and not any one single fraction of oil (Lambert *et al.* 2003). Chemical changes thereby occurring within any of the oil fractions within this broad range can still yield the same TPH whereby a reduction in one fraction may be counterbalanced by an increase in another fraction. It is possible that the chemical composition within the dispersants was altered, but these changes were not detected by the fluorometer. GC-MS analysis conducted post-exposure would obviously be beneficial to confirm whether there is an actual loss of TPH, or alteration to the chemical composition of the oil, over the exposure period, especially where the $\Delta F/F_m'$ of the seagrass is clearly impacted, but this was outside the scope of this study.

The benefits of using a semi-quantitative measurement of TPH are still apparent from the results of this study. Apart from logistical simplicity, the method was capable of measuring differences between the pre-exposure WAF concentrations i.e. the fluorometer produced increasing TPH values with increasing WAF concentrations. In most cases these pre-exposure TPH values, determined by the semi-quantitative method, coincided with the level of impact to the seagrass i.e. the greater the TPH, the greater the impact to the seagrass. Furthermore, apart from the dispersant alone treatments, the level of impact to the seagrass generally coincided with the percent TPH remaining at

the conclusion of the experiment i.e. the greater loss of TPH over the exposure period, the greater the impact to the seagrass.

In this suite of experiments, the seagrasses were exposed to the petrochemical treatments for five hours. In some of the treatments, impacts were detected within the first hour of exposure, but in most, especially those where a significant impact was still detected at the conclusion of the experiment; the main impact was not detected until several hours exposure. Furthermore, some treatments led to an elevation in $\Delta F/F_m'$ of the seagrass, most commonly within the first hour. With these considerations in mind, it appears that a laboratory testing protocol would need to run for at least several hours to a) overcome initial stimulatory effects of the petrochemicals to the seagrass; and b) to enable sufficient time for photosynthetic impacts to be detected.

Obviously these experiments do not account for the multitude of biotic and abiotic factors that determine the toxicity of oil or dispersed oil on an organism (Clark & Noles 1994; NRC 2005). This protocol was an attempt to simplify these confounding factors that occur in a real spill situation. Perhaps the limiting factor for oil spill mitigation managers is that research tries to replicate every possible abiotic and biotic variable coupled with the particular type and volume of oil in any one area. It is suggested that attempting to address every possible situation simply leads to confounding the information that research is producing.

This protocol addresses the impacts of oil and dispersed oil on seagrass in a rapid and logistically simple laboratory situation. The main method of detection for the photosynthetic impact to the seagrass, assessing the effective quantum yield, provided a rapid assessment technique. The pigment analyses in these experiments detected only few impacts to the photosynthetic health of the seagrass even when the fluorometric analyses showed clear and in some cases very severe declines in the $\Delta F/F_m'$. Macinnis and Ralph (2003a) suggested the fluorometric technique was far more sensitive in detecting photosynthetic impact than chlorophyll pigment assessment when *Z. capricorni* were exposed to petrochemicals. It is difficult to find a secondary assessment measure of seagrass health to match the speed and sensitivity of detection that PAM fluorometry provides. Other assessments of seagrass health such as oxygen production

and consumption may be valid for petrochemical testing (as used by Hatcher & Larkum 1982) but would increase the logistical difficulty when the main aim for the protocol is to be rapid and simple in design. However, oxygen production and consumption, and other analytical techniques may need to be addressed in future developments of the rapid testing protocol.

The experimental protocol developed in this study can be altered depending on the type and amount of oil spilt, the dispersant type being considered, the seagrass species in question, seawater temperature and light levels. These variables can be made-to-fit the spill in question. Obviously the toxicity of the petroleum hydrocarbons to seagrass will need to address the multitude of abiotic and biotic variables, but very little research can provide such definitive answers when they are needed most, when an oil spill occurs.

Laboratory experiments can never replicate exactly what will occur in real field conditions, but they can provide guidance as to what may occur. Considering the lack of available information on seagrass response to oil spills and the associated dispersant usage, these experiments will assist with mitigation response. The next step in this work is to determine whether these results reflect those of the field experiments and the whole plant laboratory experiments. Validation of this laboratory work will provide greater opportunity to use this data to benefit oil spill clean up managers resulting in less net environmental damage.

6 General Discussion

The primary aim of this study was to assess whether subtidal seagrass were impacted by non-dispersed and dispersed oil. A secondary aim was to assess whether field results could be replicated under laboratory conditions. The first part of this discussion thereby addresses these two main aims by summarising the results of all experiments under all conditions and for all species investigated. The latter part of the discussion expands upon the interpretation of these results in light of other factors.

6.1 Summary of Field and Laboratory Results

All chlorophyll *a* fluorescence data ($\Delta F/F_m'$) from the field and laboratory experiments have been synthesised into Tables 6.1 and 6.2 for summary purposes and to enable a comparison of the results. To differentiate between the levels of impact, the magnitude of decline in $\Delta F/F_m'$ from the control has been classified as "Low" (< 0.15), "Medium" (0.15 – 0.30) and "High" (> 0.30). A "High" level effect (> 0.30 units $\Delta F/F_m'$) represents a reduction of greater than 50 % of the photosynthetic efficiency of the seagrass. The classification does not take into account where the seagrass did not recover from a photosynthetic impact as a result of the petrochemical treatment. Recovery following treatment, or lack of it, is highlighted in the text. The timing of impact details any sampling time within the exposure and recovery periods (where applicable) when a treatment significantly decreased in $\Delta F/F_m'$ from the control. The range of concentrations that caused these significant decreases in $\Delta F/F_m'$ is included and is derived from the total petroleum hydrocarbon (TPH) concentration pre–exposure as detected by Gas-Chromatography Mass Spectrometry analyses (Chapter Two). The 2.00 % water accommodated fraction (WAF) samples are represented as being greater than (>) the TPH of the 1.00 % WAF treatment. The 1.00 % WAF treatment was produced by adding 50 g oil to 5 L seawater, and all WAF concentrations below this were produced via a dilution of the 1.00 % WAF treatment. The 2.00 % WAF treatment, however, was produced by adding 100 g oil to 5 L seawater. As the 2.00 % WAF treatment was created using a different loading regime, the TPH concentration cannot

be calculated from the 1.00 % WAF treatment (Singer *et al.* 2000). Stimulation of growth (hormetic responses) occurred in most treatments and as these did not result in a negative impact during the experiment they have simply been marked with an *. For example, "No negative impact *" denotes that the $\Delta F/F_m'$ of the seagrass may have increased above the control at some sampling time from at least one concentration, at no time did any of the samples exhibit a decrease in $\Delta F/F_m'$ from the control.

Tapis crude oil: non-dispersed, dispersed and dispersants alone

Tapis crude oil (non-dispersed)

The results from these experiments indicate no more than a minor negative impact when *Z. capricorni* and *H. ovalis* were exposed to the Tapis crude oil up to 2.00 % WAF; and to *Z. muelleri* up to 0.40 % WAF (Table 6.1). Whilst these results are in clear contrast to Macinnis and Ralph (2003a) who found the $\Delta F/F_m'$ of *Z. capricorni* decreased with exposure to crude oil, and remained depressed even following the replenishment of 'fresh' seawater; the findings are in agreement with those of Hatcher and Larkum (1982), *Posidonia australis*; Durako *et al.* (1993), *H. ovalis*, *H. stipulacea* and *Halodule uninervis*; and Ralph and Burchett (1998a), *H. ovalis*; all of whom found no significant, or only minimal, impacts from exposure to crude oil. In this study, the $\Delta F/F_m'$ of the seagrass decreased no more than 0.15 units (~ 25 %) below that of the control, and there were no significant differences detected between the chlorophyll *a* pigment concentrations, for all species, in all experiments. The crude oil comprised about 80 % of hydrocarbons within the C_6- C_9 range, mostly consisting of the BTEX (benzene, toluene, ethyl benzene, xylene) hydrocarbons (Chapter Two). These components are usually lost quite rapidly following weathering and should have been largely depleted within a few hours following the initial exposure (Dodd 1974; NRC 2005). While this appeared the case in all *in situ* experiments, the laboratory experiments (whole plants and leafblades) showed little difference between the pre- and post exposure measurements. These differences in the percentage total petroleum hydrocarbons (TPH) recovered in the *in situ* compared with the laboratory experiments were clearly not corroborated with any large differences in the photosynthetic stress to the seagrass between the experiments.

Table 6.1: Summary table of magnitude of stress ($\Delta F/F_m'$), timing of impacts and effective concentrations in *Z. muelleri, Z. capricorni* and *H. ovalis* from exposure to the Tapis crude oil treatments (non-dispersed, dispersed and dispersant alone). * not significantly different to control. na treatment was not performed under those conditions. See text for further explanation.

	Field Experiments		Laboratory Experiments			
	Z. muelleri	*Z. capricorni*	*Z. capricorni*	*Z. capricorni*	*Z. capricorni*	*H. ovalis*
Treatment	Summer	Summer	Winter	Whole plant	Leafblade	Whole plant
Tapis	No negative impact *	No negative impact *	**Low 6-8, 48 h 2.4-4.8 mg L^{-1}**	No negative impact	**Low < 1-3 h >12 mg L^{-1}**	No negative impact
Tapis + C9527	No negative impact *	**Medium 4, 24 h 20-40 mg L^{-1}**	**Medium 4 h 40 mg L^{-1}**	**Low 6-8 h >101 mg L^{-1}**	**High < 1-5 h 10 >101 mg L^{-1}**	**Medium 2h >101 mg L^{-1}**
Tapis + Ardrox	Na	na	na	**Medium 8-72 h >105 mg L^{-1}**	**Medium <1, 3-5 h 42 >105 mg L^{-1}**	**Medium 2h >105 mg L^{-1}**
C9527	Na	No negative impact	**Medium 4 h 64 >128 mg L^{-1}**	**Medium 2, 6-8, 24-96 h 32 >317 mg L^{-1}**	**Medium 3-5 h 64 >317 mg L^{-1}**	**Medium 48-96 h >317 mg L^{-1}**
Ardrox	Na	na	na	**Medium 4-48, 96 h 92 >230 mg L^{-1}**	**Medium < 1- 5 h 92 >230 mg L^{-1}**	**High 2-4, 24-96 h 92 >230 mg L^{-1}**

Tapis crude oil dispersed with Corexit 9527

In the whole plant experiments, the crude dispersed with Corexit 9527 did not result in any great decrease in the $\Delta F/F_m'$ of *Z. capricorni* (field and laboratory), *Z. muellleri* (field) or *H. ovalis* (laboratory) with only short-lived negative impacts detected in all species. Similarly, Ralph and Burchett (1998b) also reported minimal declines in $\Delta F/F_m'$ of *H. ovalis* when exposed to a combination of aged Bass Strait crude oil and Corexit 9527 with the seagrass exhibiting full recovery by the end of the experiment. Thorhaug *et al.* (1986), found 100 % mortality in the tropical seagrass *Halodule wrightii* and *Syringodium filiforme* after only five hours exposure to Corexit 9527 dispersed Louisiana crude oil. In the same study, however, *Thalassia testudinum* showed only 47 % mortality from the same treatment, providing evidence of a difference in resilience to petrochemicals amongst seagrass species (Thorhaug *et al.* 1986). In the current study, the finding that the whole plant experiments of all seagrass investigated (*Z. capricorni*, *Z. muelleri* and *H. ovalis*) showed little impact suggests that these temperate species (and subspecies) are in fact quite resilient to Corexit 9527 dispersed Tapis crude oil under these conditions.

In the leafblade experiments in this study, however, the $\Delta F/F_m'$ of *Z. capricorni* exhibited a severe level of stress and a clear lack of recovery, a far greater effect in terms of severity and speed of impact above that of both summer and winter *in situ* experiments, and the whole plant experiments in the laboratory. The reduced exposure period in the leafblade experiments compared with the whole plants (five hours compared with ten hours) implied there should be more TPH remaining in these leafblade experiments than in the whole plant experiments, however, the recovered TPH concentration in the leafblades was approximately 20 % less than that recovered in the whole plant experiments. Considering the severity of impact in the leafblade experiments, there is an implication to suggest a link between the amount of oil 'lost' and the photosynthetic stress to the seagrass. The use of Vaseline as a barrier to prevent petrochemical hydrocarbon ingress into the open wound was trialled prior to the use in these experiments. Seagrass blades with open wounds (no Vaseline) displayed greater reductions in EQY than those with Vaseline sealing the wound. Future research into this

technique, however, would need to validate whether a complete exclusion of petrochemical hydrocarbons with the use of Vaseline on the open wound does indeed occur. One method to validate this would be an assessment of the hydrocarbon concentration within the leafblade itself, which is discussed in detail later in this discussion. As with other treatments, and discussed later within this chapter, the results of the chlorophyll *a* pigment concentrations did not support this amplified level of stress in the $\Delta F/F_m'$ data in the leafblades as no significant differences were detected.

Tapis crude oil dispersed with Ardrox

Under laboratory conditions, the crude oil dispersed with Ardrox caused significant declines to the $\Delta F/F_m'$ of *Z. capricorni* in both the whole plants and leafblade experiments. This treatment was not exposed to the seagrass *in situ*. Following five hours exposure, the leafblades showed no signs of recovery which may provide some indication of the prolonged impact that occurred in the whole plant experiments. Although the magnitude of depression in $\Delta F/F_m'$ was the same between the two experiments ('medium', Table 6.1), the effect in the leafblade exposures was from lower concentrations and with less exposure time than that in the whole plant experiments. Furthermore, significant differences were evident in the chlorophyll *a* pigment concentrations in the leafblade experiments of *Z. capricorni*, but not in the whole plant exposures.

Although *H. ovalis* has been shown as one of the most sensitive species from studies investigating UV elevation (Dawson & Dennison 1996), light inhibition (Cheshire *et al.* 2002) and exposure to other pollutants, such as herbicides (Haynes *et al.* 2000), there was little evidence to support this sensitivity from this treatment, nor the crude oil alone, or crude dispersed with Ardrox treatment. Rather, *H. ovalis* appeared quite tolerant to these treatments and this finding agrees better with the findings of Ralph and Burchett (1998b). In the laboratory whole plant experiments, *H. ovalis* exhibited a short-lived (2 h only) stress response in $\Delta F/F_m'$ and displayed significant differences in the chlorophyll *a* pigment concentrations at ten hours. Obviously some alteration to the photosynthetic

output of this species has occurred but there was little evidence of a prolonged impact during the recovery period.

There appears to be a clear difference between the effects this petrochemical treatment has on the two morphologically different species used in these experiments, as *H. ovalis* appeared more tolerant than *Z. capricorni* as the affects to the latter lasted from eight hours exposure up to three days following the replenishment of 'fresh' seawater. Both species of seagrass were exposed to the same petrochemical treatments in the same tank so there is no suggestion that differences between the preparations of the WAF treatments occurred. It was, however, only the 2.00 % WAF treatment (> 105 mg L^{-1} TPH) that led to impacts to the whole plants of both species, and these concentrations represent a worse case scenario of oil concentration in subtidal regions (George-Ares & Clark 1995; Reddy & Quinn 1999).

The TPH recovered in the whole plant experiments was 10 to 20 % lower than that recovered in the leafblade experiments. Differences between the rates of petrochemical breakdown between the two experiments would likely have occurred due to differences in partitioning of the oil and accumulation into the sediments, discussed later. About 50 % of the total TPH of this treatment comprised hydrocarbons within the C_{10} to C_{14} range. It is this range of hydrocarbons that Clark *et al.* (2001) suggested poses the greatest risk to organisms due to the high toxicity coupled with the longer residence time in the environment compared with the highly toxic lower molecular weight counterparts which are lost rapidly. However, the crude dispersed with Corexit 9527 had a greater concentration of these components (C_{10} to C_{14}) than the crude dispersed with Ardrox yet resulted in a similar, short-lived (2h) response from *H. ovalis*, and actually less of an affect to *Z. capricorni*. The TPH and BTEX concentrations of the 1.00 % Ardrox dispersed crude WAF treatment (105 and 12 mg L^{-1} respectively) were slightly greater than that within the Corexit 9527 dispersed crude WAF treatment (101 and 11 mg L^{-1} respectively). Although these differences in the TPH and BTEX concentrations between the two 1.00 % WAF treatments appear minor, they may suggest a reason for the difference in the level of impact detected in the seagrass. The difference between the concentrations of both TPH and BTEX within the 2.00 % WAF treatments between the dispersed crude oil treatments is likely to have been far greater

than that which was evident between the 1.00 % WAF treatments. Unfortunately, the 2.00 % WAF treatments were not quantitatively analysed and as they were produced under a different loading regime to the 1.00 % WAF treatment cannot be calculated from the 1.00 % WAF treatment (see Chapter Two, Singer *et al.* 2000). However, as the 2.00 % crude dispersed with Ardrox WAF treatment was the only concentration to elicit a negative response in both *Z. capricorni* and *H. ovalis*, it is speculated that a greater concentration of TPH or more specifically, BTEX components may have led to the different toxicities of the two treatments.

Corexit 9527 alone

In the current study, Corexit 9527 was toxic to the seagrass, but this was more, or only, evident under the laboratory conditions. Corexit 9527 is considered to have low to moderate (LC/ EC_{50} 1 >100 ppm) acute toxicity to most aquatic organisms in laboratory tests (George-Ares & Clark 2000). However, this information is largely derived from toxicity tests on animals rather than plants, and where plants have been investigated they are on early life-stage forms, such as zoospores (eg. Burridge & Shir 1995). With regards to seagrass exposure to Corexit 9527, Thorhaug *et al.* (1986) found the dispersant toxic to some tropical species whilst Ralph & Burchett (1998b) found minor reductions to temperate *H. ovalis*.

The response of *Z. capricorni* to Corexit 9527 in this study is similar, although of a greater magnitude, to that described by Macinnis & Ralph (2003a) when the same species was exposed to the dispersant VDC. In both studies, the current study and Macinnis and Ralph (2003a), the seagrass showed a greater negative response in laboratory experiments than that observed *in situ*, and the greatest stress response was detected following the replenishing of 'fresh' seawater. In the laboratory experiments in the current study, this stress was quite severe with all concentrations showing significant decreases in $\Delta F/F_m$' and an increase in stress response during the recovery period. Similarly, the $\Delta F/F_m$' of *H. ovalis* indicated an increased stress response in the recovery period although from fewer concentrations than *Z. capricorni*. This delayed impact, also evident in other dispersant alone treatments (eg. Ardrox and Corexit 9500),

may be due to the adherence of the dispersants to the seagrass resulting in some dispersant remaining even following the replenishment of the 'fresh' seawater. Whilst this may appear to be an experimental artefact, it could easily replicate a real spill event. If a dispersant is over-applied to an oil slick and the weather conditions lead to penetration of the dispersant into the subtidal seagrass meadow, the effects to the seagrass meadow could be quite severe. However, the TPH recovered in the laboratory experiments was high suggesting that most of the dispersant did, in fact, remain in the water column. This implies that the dispersant may have a delayed impact to the seagrass, and not simply related to an adherence to the blade.

The $\Delta F/F_m'$ in the leafblade experiments correlated well with the whole plant laboratory exposures with the lack of recovery at the conclusion of the leafblade experiments somewhat indicative of the recovery day impacts. The leafblades did exhibit an amplification of the effects to the seagrass compared to the whole plant exposures but only through more concentrations in the leafblades causing significant differences. Furthermore, there were significant differences detected in the chlorophyll *a* pigment concentrations in the leafblades, but not in the whole plants; due to an increase in chlorophyll *a* concentration from the lowest WAF treatment, which had also shown an elevation in the $\Delta F/F_m'$.

There was no real difference in TPH recovered between the two laboratory experiments. The total TPH of this treatment (300 mg L^{-1}, 1.00 % WAF) was the highest for all the crude treatments used in this study (Chapter Two). The treatment comprised equally C_{10} to C_{14} and C_{15} to C_{28} hydrocarbon fractions which accounted for more than 90 % of the total sample. The longer residence times of these hydrocarbon fractions, compared with the lower weight hydrocarbon fractions (Dodd 1974), may account for the high recovery rates of this treatment following the exposure period.

Ardrox alone

Ardrox was toxic to both *Z. capricorni* and *H. ovalis*, and similar to the Corexit 9527 alone treatment, and the dispersant VDC in Macinnis and Ralph (2003a), caused an

increase in stress response after the ten-hour exposure period in the whole plant experiments. The $\Delta F/F_m'$ of the leafblades of *Z. capricorni* showed no recovery at the end of the five hour exposure and is possibly indicative of the continued impacts during the recovery days in the whole plant exposures. There was also an earlier detection of impact in the leafblade experiments than the whole plant experiments implying the benefits of the testing protocol as a rapid detection measure. Although the $\Delta F/F_m'$ data detected severe stress to both species, the chlorophyll *a* pigment concentrations, again, detected no significant differences when the seagrass was exposed to the treatment. The percentage TPH recovered was quite high for this treatment, with the whole plants only slightly greater than that which was recovered in the leafblade experiments. The GC-MS analysis found this treatment to contain less TPH than the Corexit 9527 due to a reduction in the concentration of C_{10} to C_{14} hydrocarbons. This is interesting as the Ardox alone treatment caused a greater stress to the $\Delta F/F_m'$ of the seagrass than the Corexit 9527 alone treatment, yet contained fewer hydrocarbons. Both Ardrox and Corexit 9527, had very little concentrations of BTEX and PAHs, so it appears to be some other confounding factor, other than hydrocarbon concentration or composition that is directing this impact. A comprehensive chemical analysis of the water column and of the seagrass blade post-exposure may help in addressing what is occurring.

Although this treatment was exposed to the seagrass under laboratory conditions only, the magnitude and prolonged duration of impact clearly warrants cause for concern to seagrass when this dispersant is used in a real spill situation. It is strongly recommended that further research is directed at addressing the effects this dispersant has on subtidal seagrass species considering its application in recent oil spill events (eg. Montara Platform oil leak (AMSA 2009)).

IFO-380: non-dispersed, dispersed and dispersants alone

IFO-380 (non-dispersed)

The results from this study suggest IFO-380 leads to minor photosynthetic stress in *Z. capricorni* and *H. ovalis* up to 2.00 % WAF, over a ten hour exposure period (Table

6.2). For *Z. capricorni,* minor impacts were detected only in the winter *in situ*, and the leafblade experiments, with similar magnitudes of effect in all experiments, < 0.15 units below the control ("Low" level of impact). *Halophila ovalis* showed a slightly greater photosynthetic stress than *Z. capricorni* with regards to the level of decline in $\Delta F/F_m'$, and also the oil concentrations that exhibited this decline; but the effects were short-lived. The chlorophyll *a* pigment concentrations of *H. ovalis* in the oil treatments were significantly lower than that of the control at 96 hours which clearly does not support the $\Delta F/F_m'$ data. It remains unclear why this was so, yet is similar to the findings from other treatments where the two photosynthetic parameters did not show agreement.

The lack of hydrocarbons remaining at the conclusion of the *in situ* exposure period in both seasons was not corroborated by the amount recovered in the laboratory experiments for the same concentrations. There appeared no relationship, however, between the amount of TPH recovered and the level of photosynthetic stress to the seagrass between the experiments. It is likely that higher rates of microbial activity within the water column and sediments *in situ* compared to the laboratory exposures influenced the breakdown and partitioning of the petrochemicals (Leahy & Colwell 1990; Clark & Noles 1994) rather than any differences in incorporation of the oil into the seagrass itself.

IFO-380 dispersed with Slickgone

The two species, *Z. capricorni* and *H. ovalis*, responded differently to the IFO-380 dispersed with Slickgone treatment. *Halophila ovalis* was impacted more severely than *Z. capricorni* in the exposure period and extending into the recovery days, exemplifying the range of response amongst seagrass species documented by Thorhaug *et al.* (1986). There was no negative effect to *Z. capricorni* from the IFO-380 dispersed with Slickgone treatment in either the summer *in situ* or in the whole plant laboratory experiments, supported by the results of the $\Delta F/F_m'$ and the chlorophyll *a* pigment analyses. The effect to *Z. capricorni* in winter *in situ* compared with the summer experiment may be caused by the difference in seagrass growth in the two seasons, rather than a difference in the rate of petrochemical breakdown. Larkum *et al.* (1984)

found a four-fold reduction in shoot biomass in winter in *Z. capricorni* in Botany Bay compared with summer and species have been shown to be more severely impacted by stress in different seasons (eg. Brun *et al.* 2002). It is suggested that there was no great difference between the rates of breakdown of the petrochemicals as the amount of TPH recovered was similar between the two seasons. The medium level impact in the winter *in situ* experiment was, however, short-lived, lasting no more than four hours and there were no significant differences detected in the chlorophyll *a* pigment concentration. Interestingly, the winter *in situ* and the leafblade exposures showed the same level of effect to the $\Delta F/F_m'$ of *Z. capricorni* from the 0.40 % WAF concentration, whilst in the leafblades, the 1.00 and 2.00 % (not performed *in situ*) concentrations also negatively effected the seagrass. This agreement between the leafblades and the winter *in situ* suggests the leafblade experiments are overestimating the impacts to what is observed under summer conditions and may be useful as an early detection of stress and for rapidly detecting differences between different mitigation strategies.

IFO-380 dispersed with Corexit 9500

The IFO-380 dispersed with Corexit 9500 resulted in quite severe declines in the $\Delta F/F_m'$ of both *Z. capricorni* and *H. ovalis*, but was exposed to the seagrass under laboratory conditions only. There were also significant differences in the *H. ovalis* chlorophyll *a* pigment concentrations. Although there was an increase in stress to *Z. capricorni* once the exposure tanks were replenished with 'fresh' seawater, the seagrass had recovered by the end of the experiment. When the IFO-380 dispersed with Corexit 9500 was exposed to the leafblades of *Z. capricorni*, the impacts were detected earlier, were more severe and from lower concentrations than in the whole plant exposures. Similar to other treatments where the leafblades overestimated the impacts observed in the whole plants, this early detection of impacts may be useful in the rapid assessment protocol. Furthermore, the $\Delta F/F_m'$ in the leafblade experiments was clearly decreasing at the conclusion of the experiment and similar to other treatments, which may be indicative of prolonged or delayed impacts seen in the recovery period of the whole plant experiments in the laboratory. Although this treatment was only conducted under laboratory conditions, the severity of the impact from this, and the Slickgone dispersed

treatment suggests that mitigation procedures using dispersants on IFO-380 may be more stressful to the seagrass meadow than mitigating an oil spill via other means.

The TPH of the 1.00 % WAF of this treatment was 500 mg L^{-1}, the highest concentration recorded in any of the WAF treatments, and more than twice as high as the Slickgone dispersed IFO-380. Furthermore, compared with other treatments, this treatment contained far higher concentrations of the polycyclic aromatic hydrocarbons, naphthalene (320 mg L^{-1}) and phenanthrene (560 mg L^{-1}) (Table 2.1), and also pyrene (220 mg L^{-1}), benzo(a)anthracene (110 mg L^{-1}) and chrysene (160 mg L^{-1}) (data not shown). As polycyclic aromatic hydrocarbons (PAH) are considered highly toxic to organisms (Maki *et al.* 2001; Lee 2003; Kirby *et al.* 2007), the toxic potential of this treatment was clearly different to the other treatments. The high TPH concentration coupled with the relatively high concentrations of PAHs may be a reason for the higher level of impacts of this treatment when compared to the other treatments, including the IFO-380 dispersed with Slickgone.

Slickgone alone

Exposing *Z. capricorni* and *H. ovalis* to the Slickgone alone treatment resulted in, at worst, only minor impacts. For the Slickgone alone treatment, there were no negative impacts to *Z. capricorni* with the results of the whole plant laboratory experiments and the *in situ* experiments in both seasons supporting these findings, and only minor impacts to the species in the leafblade experiments. There were impacts detected in the chlorophyll *a* pigment analyses of *Z. capricorni*, but these were only detected in the leafblade experiments. Similarly, *H. ovalis* showed little stress as impacts were detected at only one sampling time during the exposure period and there were no differences in the chlorophyll *a* pigment concentrations. Whilst the TPH of the 1.00 % Slickgone alone WAF treatment, was similar to that of the Corexit 9500 alone treatment, there was a greater concentration of BTEX hydrocarbons in the Corexit 9500 alone treatment than this treatment. This may help to explain the difference in the levels of stress to the seagrass from these two dispersant alone treatments. Although, these results suggest limited stress to both species from this treatment, the combined effect of this dispersant

with the IFO-380 was more severe to both species, but especially to *H. ovalis*, which should be considered in mitigation procedures when the two species occur within the same vicinity.

Corexit 9500 alone

Similar to Corexit 9527, the dispersant Corexit 9500 has been described as having low to moderate acute toxicity (LC/ EC_{50} 1 >100 ppm) to most aquatic organisms, mainly animals (George-Ares & Clark 2000). This, however, does not represent the response of seagrass found in this study. Although this treatment was exposed to the seagrass in laboratory experiments only, it led to the greatest reduction in $\Delta F/F_m'$ in both *Z. capricorni* and *H. ovalis*. The $\Delta F/F_m'$ decreased below 0.4 units of that of the control in both the exposure and recovery periods in *H. ovalis* and below 0.3 units of the control in *Z. capricorni* during the recovery period. Interestingly, there were significant declines in the chlorophyll *a* pigment concentrations of both species in all experiments. This was the only treatment that caused such comprehensive declines in the chlorophyll *a* pigment concentrations in all experiments and is highly supportive of the severity of impact as detected in the $\Delta F/F_m'$ results. The prolonged and delayed impact is clearly concerning when this dispersant is considered for use over areas of subtidal seagrass. Although the *Z. capricorni* leafblade experiments did not detect this level of impact, it is suggested this was due to a delayed impact from this treatment as evident in the whole plant experiments. The five-hour exposure period in the leafblade experiments was somewhat lacking with regards to detecting the severity of impact from this treatment; however, there was some pattern of decrease up until the end of the five hours exposure which may suggest an increased level of impact had the experiment run for a longer period of time. This, along with similar indications from other treatments, suggests that any continual decrease, or lack of recovery, towards the end of the five hour exposure period should be carefully evaluated and mitigation managers would need to proceed with caution.

Table 6.2: Summary of magnitude of stress ($\Delta F/F_m'$), timing of impacts and effective concentrations in *Z. capricorni* and *H. ovalis* from exposure to the IFO-380 treatments (non-dispersed, dispersed and dispersant alone). * not significantly different to control. na treatment was not performed under those conditions. See text for further explanation. #Tukeys showed no significant difference.

	Field Experiments		Laboratory Experiments		
	Z. capricorni	*Z. capricorni*	*Z. capricorni*	*Z. capricorni*	*H. ovalis*
Treatment	Summer	Winter	Whole plant	Leafblade	Whole plant
IFO-380	No negative impact *	**Low 2-4, 48 h 1 mg L^{-1}**	No negative impact *	**Low <1, 4-5 h 3 >3 mg L^{-1}**	**Medium 2-4 h 1 >3 mg L^{-1}**
IFO-380 + Slickgone	No negative impact *	**Medium 2-4 h 80 mg L^{-1}**	No negative impact	**Medium < 1-5 h 80 >200 mg L^{-1}**	**Severe 4-8, 24, 96 h 80 >200 mg L^{-1}**
IFO-380 + C9500	Na	na	**Medium 6-48 h 200 >500 mg L^{-1}**	**High 3-5 h 100 >500 mg L^{-1}**	**Medium 2-6, 10-24 h 200 >500 mg L^{-1}**
Slickgone	No negative impact *	No negative impact *	No negative impact	**Low 4-5 h 150 >150 mg L^{-1}**	**Medium 8 h >150 mg L^{-1}**
C9500	Na	na	**Severe 24-48 h 67 >167 mg L^{-1}**	**Low < 1, 3-5 h 167 >167 mg L^{-1}**	**Severe 2-48, 96 h >167 mg L^{-1}**

The mixture of IFO-380 and Corexit 9500 had a far greater TPH concentration than the dispersant alone yet resulted in a lesser impact to the seagrass. The Corexit 9500 alone treatment had the highest concentration of BTEX (benzene, toluene, ethylbenzene and xylene) hydrocarbons (820 mg L^{-1}) compared with any of the IFO-380 treatments and this was twice that of the dispersant and oil mixture. This may suggest why the dispersant alone had a greater impact than the dispersed oil treatment. These hydrocarbons have been well documented as causing severe stress to organisms (Capuzzo & McDowell 1987; Herman *et al.* 1991) but, the author could not find any studies that have investigated their effect on seagrass. Although, the crude treatments had higher concentrations of these components, the severity of impact that the Corexit 9500 treatment caused to the seagrass and that it did not appear related to the TPH concentration may warrant cause for further investigation into this.

There was very little TPH 'lost' over the exposure period and some treatments actually showed an increase above that of the pre-exposure measurement. It is possible that chemical alterations within this treatment over the exposure period had occurred but as discussed previously, may simply be an inability of the oil-in-water fluorometer to accurately detect and measure these differences (Lambert *et al.* 2003).

6.2 Replication of Results Between Field and Laboratory

While laboratory experiments are thought to exaggerate the results compared with those obtained *in situ*, in this study this was true for some treatments only. In some cases, the laboratory results underestimated the impacts to the seagrass. The leafblades, however, exhibited a greater negative photosynthetic response than both field and whole plant exposures in the laboratory in some of the treatments. The leafblades of *Z. capricorni* exhibited either an earlier onset of effect or an effect, due to a greater range of concentrations than the whole plants for most of the crude oil treatments (dispersed and non-dispersed).

The two oil alone treatments, crude oil alone and IFO-380 alone, showed similar findings between those experiments conducted under summer conditions (summer *in situ*, and laboratory whole plants and leafblades). The winter *in situ* experiments displayed a greater impact for these two oil alone treatments suggesting possible seasonal differences in seagrass metabolism (Masini and Manning 1997; Bostrom *et al.* 2004), or differences in the rate of petrochemical breakdown due to varying environmental conditions (NEMR 2000; Singer *et al.* 2000).

Comparison of results between the experiments with regards to the pigment concentrations is difficult. The chlorophyll *a* pigment analysis detected few differences between the petrochemical treatments and controls in all experiments and showed little correlation with the effects detected in the $\Delta F/F_m'$ data. There was more evidence of impact in the leafblade samples than the whole plants (field and laboratory) of *Z. capricorni*. The only time where the pigment concentrations showed good correlation between experiments was in the *Z. capricorni* laboratory whole plant and leafblade experiments with exposure to Corexit 9500 alone. Macinnis and Ralph (2003a) detected changes in chlorophyll *a* pigment concentrations when exposing *Z. capricorni* to non-dispersed and dispersed crude oil, and to dispersant alone. They found when the seagrasses were exposed to the chemically dispersed oil, the laboratory samples were more sensitive than the field, whereas field samples were more sensitive than the laboratory samples, when the seagrass was exposed to the crude oil alone treatment. However, no such general findings were apparent in this study due to the low incidence of significant differences.

Clear differences were detected in this study regarding the amount of TPH recovered between the field and laboratory experiments. In the *in situ* experiments, there was minimal, if any, TPH recovered following the exposure period in most treatments which contrasted with the relatively high levels of TPH recovered following the same duration of exposure in the laboratory experiments. There was good correlation between the TPH recovered in the laboratory, whole plant and leafblade, experiments even when there was a substantial loss of TPH (eg. crude dispersed with Ardrox, IFO-380 dispersed with Slickgone, IFO-380 dispersed with Corexit 9500) and even though the exposure period in the whole plant experiments ran for twice as long as the exposure period in the

leafblades. As sediments and microbial activity play a major role in the breakdown of petrochemicals (Leahy & Colwell 1990; Page *et al.* 1999; Fingas 2001), a reduction of these components in these in laboratory experiments (Clark & Noles 1994) has led several authors to suggest that an increase in photosynthetic stress to organisms in laboratory experiments may be derived from a reduced rate of petrochemical breakdown by these microorganisms (eg. Macinnis & Ralph 2003a). In the whole plant laboratory experiments in the current study, there would have been fewer microbes because of the filtered seawater, and due to the reduced amount of sediment when compared with the *in situ* experiments. Loss of TPH via evaporation should have been minimal, if at all, as all exposure tanks (mesocosms, aquaria and sample jars) were sealed with some form of lid and there was minimal, if any, headspace. If anything, the whole plant laboratory experiments may have had slightly elevated rates of evaporation of TPH as there was a small headspace in these exposure tanks, whereas *in situ* there was no headspace. Considering these factors, it is likely that the greater loss of TPH in the laboratory experiments compared with the *in situ* experiments is due to the reduced microbial activity and sediments in the laboratory experiments.

For certain treatments, the detection of photosynthetic stress in the seagrasses *in situ* was similar to that detected under the laboratory conditions. This is largely based on the results of the $\Delta F/F_m'$ data as the apparent lack of sensitivity in the chlorophyll *a* pigments enables few comparisons between the experiments. The whole plant laboratory experiments, conducted under summer conditions, replicated those observed in summer *in situ* experiments for most treatments. More stress was detected in winter *in situ* compared to the whole plant laboratory experiments for crude alone, IFO-380 alone, Corexit 9527 alone, and IFO-380 dispersed with Slickgone. The leafblade results overestimated those observed *in situ* in summer for all treatments; and for most treatments in the whole plant laboratory experiments; but were similar to those observed in the winter *in situ* experiments for most treatments (eg. crude alone, Corexit 9527, IFO-380 alone, IFO-380 dispersed with Slickgone). The overestimation of impacts in the leafblade experiments compared with the whole plants under the same condition (temperature) suggests the testing protocol may be beneficial in rapidly detecting petrochemically-induced photosynthetic stress in the seagrass.

6.3 Representative of Real Spill Conditions?

There were certain elements within these experiments that may not have occurred in a real spill event and as such the results reported here may underestimate or overestimate that which would occur in a real spill event. These were either necessary in the design of the experiment, or could not be controlled.

Subtidal regions are considered less likely to be impacted by oil contamination due to the short-term exposure at such depths and the increased dilution factor and weathering of oil with increasing depth. Furthermore, George-Ares and Clark (1995) suggested that short (four hour) exposures were realistic of oil exposure to aquatic organisms. However, this does not rule out situations where a constant supply of oil may occur within a subtidal region. For example, the oil over, and within, a subtidal seagrass habitat may be constantly being replenished, such as by a leak from the hull of a damaged tanker or an oil platform or refuelling pipeline (eg. Montara Oil Platform, 2009). The case of the *Thalassia* spp. that was severely impacted to the extent of habitat destabilisation following exposure to 'fresh' (non-weathered) crude oil provides clear evidence of the potential effects to seagrass from such an occurrence (Nadau & Berquest 1977). In this study, not only was the oil weathered (i.e. not fresh), it was also a single pulse of weathered oil, which would be losing its toxicity over time. The initial impacts to the seagrass from some treatments (eg. the response of *H. ovalis* to the two dispersed crude treatments) coincide with the timing of when the weathered oil was likely to be most toxic, when first exposed in the treatment tanks. This may imply that a more serious response from the seagrass, greater than that detected in this study, may occur with a continual replenishment of the oil. Although, in most cases the petrochemicals within the water column will dilute rapidly, the possibility of a continued supply of oil to subtidal regions may warrant investigation into continual exposure experiments as a precautionary measure.

Dispersant application in Australian waters is generally not considered in waters shallower than five to ten metres (AMSA 2000). At the highest tide, the mesocosms *in situ* were submerged at a depth of just over two metres. Studies suggest that the impacts

of petrochemicals on subtidal organisms, including seagrass, decreases with increasing depth (Zieman *et al.* 1984; Jacobs 1988; NRC 2005). This is due to decreased levels of hydrocarbons with increasing water depth, caused by limited solubility and the greater mixing time required for the oil to reach such depths (NRC 2005). These experiments therefore represent a worse case scenario of oil exposure in extremely shallow water.

Another factor which would have been different to real spill conditions was the alteration in UV-light penetrating through to the seagrass, caused by the materials used in the experiments. The mesocosms in the field experiments and the lids used in the whole plant experiments in the laboratory were made from Perspex. This material reduces the penetration of UV light. A reduction in the penetration of UV light would have altered the effects of the seagrass in two ways; by reducing photo-oxidation of the polycyclic aromatic hydrocarbons (PAHs) and by reducing the potential for photoinhibition in the seagrass (Macinnis & Ralph 2003a). Photo-oxidation of polycyclic aromatic hydrocarbons (PAHs) has been well documented and has been shown in many instances to increase the toxicity of PAHs (Singer *et al.* 2000; Maki *et al.* 2001; Lee 2003; NRC 2005). As a result of the reduction of UV penetration to the oils within the mesocosms and exposure tanks, the PAHs would not have photo-oxidised as they would have under real spill conditions. This may have prevented them from reaching their full toxic potential. Photoinhibition of plants can occur with increased UV exposure (Ralph *et al.* 2002). The suppression of the photosystems and increased stress can lower the photosynthetic rate of the plant and thereby alter the $\Delta F/F_m'$ (Huang *et al.* 1997). As seagrasses can be severely impacted by photoinhibition there is the possibility that the seagrass were less impacted had there been no reduction in UV-light. Further, when a plant is stressed, it is more prone to the impacts of other stressors, including petrochemicals so this may have also altered these effects (Ren *et al.* 1995; Huang *et al.* 1997). However, the depth the seagrass were exposed in was shallow, and this may have counteracted any reduced effect this reduction in UV-light via the materials (Perspex) used had on the effects to the seagrass.

6.4 Was the Testing Protocol Useful?

As a first step in the development of a laboratory testing protocol for petrochemical exposure to seagrass, the study was beneficial. Leafblades did exhibit either an earlier onset of effect, or an effect due to a greater range of concentrations than the whole plants for most of the crude oil (dispersed and non-dispersed) treatments. The finding that these leafblade experiments may overestimate whole plant results, and considering the ranking of petrochemical toxicity was similar in most (but not all) treatments, is a valuable finding, especially when a rapid comparison of petrochemical treatments is required, such as when an oil spill has occurred.

In some treatments (eg. crude + Ardrox, C9527 alone, Ardrox alone, IFO-380 + C9500, C9500 alone) where an impact was detected in the recovery day measurements in the whole plants, the seagrass in the leafblade experiments showed either continued declines or no signs of recovery at the conclusion of the experiment. Whilst this was not true for all treatments (i.e. crude alone; IFO-380 + Slickgone, and Slickgone alone) the high incidence when this did happen is considered quite useful in possibly indicating longer term effects. Obviously, further investigations are required to validate this, and also to ascertain what caused the other treatments not to follow a similar pattern.

The leafblade experiments were far less logistically intensive to conduct than the whole plants *in situ* and under the laboratory conditions. Although the leafblade experiments, overestimated the response of the whole plant experiments, they did provide a good comparative measure of the effects of oils and non-dispersed oils. Considering that George-Ares & Clark (1995) suggested that short-term (four-hour) exposures are realistic of aquatic toxicity with regards to the oil concentration once entering the marine environment, the exposure duration in these experiments appear to represent those encountered in most spill situations. The protocol, however, may also be beneficial in being run for the duration of an oil spill. By comparing the results of the leafblade protocol, with the response of seagrass in the actual spill site, a great deal of information could be determined regarding how well the experiments simulate the real event and provide further insight on how to improve the techniqe. However, the

integrity of the leafblade, having been removed from the plant, would need to be closely monitored with the use of controls. The testing protocol can be made-to-fit the variables encountered once the oil spill has occurred including the seawater where the spill has occurred (incorporating microbial activity, temperature etc), the oil type, the available and considered dispersants, irradiance levels and the species in question. The ability of a testing protocol for the rapid detection of photosynthetic stress in seagrass from the multitude of variables encountered in a specific oil spill is clearly beneficial in future oil spill research.

6.5 Future Research

The use of oil-in-water fluorometers is still evolving in oil spill research and their use in detecting hydrocarbon concentrations in samples containing dispersants is sometimes contentious (Lambert *et al.* 2003). Further research into oil-in-water fluorometers for measuring TPH concentrations in water samples will assist with the development of rapid testing protocols. Some studies suggest that the fluorescence produced from a dispersant sample is more an element of the backscattering of the UV light within the sample, rather than any hydrocarbons (eg. Lambert *et al.* 2003). The possible anomalies with regard to the dispersant samples, whereby some treatments displayed an increase in TPH after exposure may support the findings that oil-in-water fluorometers are not ideal for measuring dispersants in a water sample (Lambert *et al.* 2003). The technique was still used in this study as a rapid measure of percentage TPH remaining following exposure. Further, the UV fluorescence technique could not be performed in the laboratory experiments due to the sample volume required, 2.5 L; whereas the sample volume required in the oil-in-water fluorometer analysis was only 15 mL. With this information in mind, the results of the dispersant alone TPH analyses for the laboratory experiments, are not recommended for forming the basis of any management decisions. Further analytical validation is required and would greatly assist with the development of a rapid laboratory testing protocol.

Another area of investigation relating to the petrochemicals is their actual fate in manipulated experiments. This study provides an indication of the loss of

petrochemicals over a defined exposure period and takes us somewhat closer to understanding the dynamics of petrochemicals under experimental conditions. However, it still remains unclear where the petrochemicals go when they are no longer recovered in the analysis. There are many possible pathways for the oils once they enter these experimental systems (Leahy & Colwell 1990; Clark & Noles 1994; Page *et al.* 1999). In these experiments, the petrochemicals may have adhered to the mesocosm wall, the leaf clips, or the fibre optics; they may have been incorporated into the sediment, or taken up by microbes or epiphytic organisms; or they may have adhered to, or been incorporated into the seagrass itself. Mass balance experiments have been conducted by other researchers with the aim of determining the fate of petrochemicals (Page *et al.* 1999). Although the majority of oil is usually not recovered once it has been 'spilled', even in controlled laboratory experiments, (eg. Page *et al.* (1999) recovered between 10 and 75 % in range of experiments) the information is still valuable. Future research focusing on a quantitative mass balance assessment will provide a greater level of understanding of these petrochemicals and their interactions with seagrass.

The determination of hydrocarbon concentration within the seagrass blade would also further our knowledge of stimulatory enhancements of growth with exposure to petrochemicals. The common occurrence of an enhancement in growth of the seagrass even when exposed to the high dosage (2.00 % WAF) treatments suggests that the petrochemicals are causing some change within the seagrass. Whilst this does not appear to be a negative impact, many studies do caution what this may mean to the organism in that there is nothing to suggest this does not eventually result in a negative impact to the organism (Kefford *et al.* 2008). The increase in $\Delta F/F_m'$ in the oil and the dispersed oil treatments in *Z. muelleri* suggests something physiologically is occurring within the seagrass. Hormetic responses are common in oil spill research with low level concentrations of petrochemicals and may be linked to an assimilation and utilisation of the carbon component within the oils (Gordon & Prouse 1973; Karydis & Fogg 1980; Chan & Chiu 1985). Determining the hydrocarbon concentration within the seagrass may help to better understand this and to determine what process causes increases or decreases in growth from different concentrations of the same petrochemical treatment.

As stated throughout this thesis, the chlorophyll *a* fluorescence technique was used as it can provide a rapid and non-destructive analysis of the photosynthetic health of the seagrass (Enriquez et al. 2002). The majority of research into seagrass response from petrochemicals has not used this technique, rather assessments of mortality and, or growth measurements, were the norm (for example Thorhaug et al. 1986 assessed mortality; Dean et al. 1993 assessed growth rates and biomass). Macinnis and Ralph (2003a) stated (p. 1396) "mortality is a good indicator of the level of toxicity as it easy to detect", whilst a decrease in biomass is also simple to assess. However, with chlorophyll *a* fluorescence techniques and the impairment of photosynthetic activity it is not as clear-cut. At present there are no clear definitions of the level that photosynthetic impairment, measured by the effective quantum yield (or any other fluorescence measurement), can decrease to that will lead to definite mortality of the seagrass. In the current study, the effective quantum yield of *H. ovalis* exposed to Corexit 9500 decreased more than 0.40 units (more than 60 %) below the pre-exposure level (p.128). The seagrass in this case showed a sustained and prolonged impact in that there was little recovery following this. In comparison, Macinnis and Ralph (2003) found *Z. capricorni* to decrease below 0.1 units EQY, equating to an approximate 80 % decrease from the pre-exposure levels, yet the seagrass recovered almost completely within ten hours, and did recover back to pre-exposure levels once returned to 'fresh' seawater. Findings from the current and previous research indicate that perhaps it is a period of prolonged photosynthetic impairment below a certain level that needs to be defined to determine when the seagrass can no longer recover from. At present, however, there is no defined level or no known period of time for seagrass mortality to occur and this is a directive for future research requirements.

The loss of epiphytic organisms along the seagrass blade may lead to increased growth due to the reduction in shading by the epiphytes causing an increase in light levels reaching the seagrass blade; alternatively, an increase in light levels may cause an increased phototoxic stress (Clough & Attiwill 1980; Ren *et al.* 1994; Masini *et al.* 1995; Huang *et al.* 1997). As studies show that epiphytic organisms can be severely impacted by petrochemical exposures (Zieman *et al.* 1984; Jacobs 1988; API 1999), this is clearly likely to impact the seagrass response to the petrochemicals and should also be incorporated into future research.

Seasonal variation in organism response to stress has been reported in other studies (eg. Brun *et al.* 2002) and the results of this study suggest that there may be differences in seagrass response to petrochemicals in different seasons. The laboratory experiments assisted in defining the winter *in situ* impacts to *Z. capricorni*. *Zostera capricorni* displayed a slightly greater impact from crude alone, IFO-380 alone, and IFO-380 dispersed with Slickgone in the winter *in situ* experiments compared with the other whole plant experiments which were conducted under summer conditions (summer *in situ,* laboratory whole plants). All laboratory experiments were conducted under Sydney summer conditions. The seagrass were collected during summer, whilst the temperature of the holding tank where the seagrass were acclimated, the treatment room for the whole plant experiments and the temperature-controlled water bath for the leafblade experiments were all maintained at 20 ± 1° C. From these experimental conditions, it appears that subtidal *Z. capricorni* exposed to certain petrochemicals in winter, can be more severely impacted than those exposed under summer conditions. Brun *et al.* (2002) found a seasonal difference in seagrass response from exposure to ammonia. Future research should further investigate potential seasonal variations to seagrass response to better understand these processes and determine whether different mitigation strategies may be required in different seasons.

Difference in species response in some of the treatments was clear. The findings clearly support the research of Thorhaug *et al.* (1986), which showed seagrass response varied when exposed to the same petrochemical treatment. Considering that *Z. capricorni* and *H. ovalis* commonly occur within the same habitat, the effects to either species would need to be evaluated when mitigation procedures are under consideration. The response of *H. ovalis* to these petrochemicals was only investigated under whole plant laboratory conditions. Similarly, only two experiments were conducted on *Z. muelleri*. Further research should be conducted on the response of these species to a wide range of petrochemicals. Considering the range of responses that these species displayed in these experiments, future experiments should continue to examine multi-species response to petrochemicals.

6.6 Conclusions

A simple and valuable outcome from this study for oil spill mitigation purposes would have been no detrimental effect from either oil or dispersed oil. This would suggest that mitigation procedures designed to protect or reduce the impact to surrounding resources could be carried out with clear knowledge that neither procedure would negatively impact the subtidal seagrass. Unfortunately, for mitigation purposes this was not the case.

These experiments suggest that oil alone, IFO-380 or Tapis crude, cause less stress to subtidal *Z. capricorni* and *H. ovalis* than the dispersed oil treatments. It also suggests that the addition of certain dispersants can lead to more stress than others. Little conclusion can be drawn as to whether *Z. capricorni* was impacted more by Corexit 9527 dispersed- or Ardrox dispersed crude oil, but for *H. ovalis* both treatments elicited a similar very short-lived response in laboratory experiments. The IFO-380 dispersed with Corexit 9500, and Corexit 9500 alone was more stressful to *Z. capricorni* than the related Slickgone treatments; conversely for *H. ovalis*, the IFO-380 dispersed with Slickgone, and Slickgone alone was more stressful than the related Corexit 9500.

The results imply it is better not to disperse over an area of subtidal seagrass bed when either Tapis crude oil or 380cSt fuel oil is spilt. However, when the addition of chemical dispersant is deemed appropriate to protect other resources within the area, the seagrass may still recover depending on the dispersant used. The dispersants used in this study were commonly applied dispersants in Australian and international oil spill events so this toxicity needs to addressed when their application is sought in areas of subtidal seagrass habitat.

Appendix: Final Report to the Australian Maritime Safety Authority

This appendix is the Final Report to the Australian Maritime Safety Authority (AMSA), the Australian Research Council Industry Partner for this research, and is publicly available on the AMSA website (www.amsa.gov.au). The 'Final Report' is a summary of the work contained in the thesis and was designed to be user-friendly for oil spill managers in Australia. The 'Final Report' has been incorporated into this thesis to provide a more rapid view of the major themes, methodologies used and recommendations arising from this research.

EFFECTS OF OIL AND DISPERSED OIL ON TEMPERATE SEAGRASS: SCALING OF POLLUTION IMPACTS

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Executive Summary

This report is a summary of research conducted into the effects of oil and dispersed oil on temperate seagrass using a range of *in situ* and laboratory experiments on whole plants and seagrass leaf blade sections. Apart from assessing the effects of oil and dispersed oil on seagrass between seasons, locations, and morphologically different species, the research also investigated whether laboratory results could be indicative of those obtained *in situ*. Petrochemical treatments consisted of whole plants exposed for ten hours with a four day recovery period to a range of concentrations of the water accommodated fraction (WAF) of oil alone (Tapis crude, IFO-380 fuel oil), dispersant alone (Corexit 9527, Ardox 6120, Slickgone LTSW, Corexit 9500) and dispersed oil. Experiments were conducted in both the laboratory and *in situ*. Photosynthetic health was monitored by assessing the effective quantum yield of photosystem II ($\Delta F/F_m'$) and chlorophyll *a* pigment concentrations, whilst semi-quantitative methods of total petroleum hydrocarbon (TPH) concentration were used to determine the percent TPH remaining in the water column following the exposure period.

In most cases, the non-dispersed oils, Tapis crude and IFO-380, had less of an impact to both *Zostera capricorni* and *Halophila ovalis* than the dispersed oil treatments. Winter *in situ* experiments found slightly greater reductions of $\Delta F/F_m$' in *Z. capricorni* in most treatments compared with summer *in situ*, but generally there was minimal impact. *Zostera muelleri* exhibited a stimulatory response to both non-dispersed and dispersed Tapis crude oil in Corio Bay, Victoria (summer *in situ* only). Laboratory whole plant experiments found *Z. capricorni* was for the most part less resilient to Tapis crude oil (non-dispersed and dispersed) treatments than *H. ovalis* whereas, with exposure to IFO-380 fuel oil (non-dispersed and dispersed) *H. ovalis* was less resilient than *Z. capricorni*. Quite severe, and, or prolonged, photosynthetic stress was evident in both *Z. capricorni* and *H. ovalis* when exposed to most of the dispersant alone treatments (Corexit 9527, Ardrox 6120and Corexit 9500), however the Slickgone LTSW alone treatment caused only a very short-lived response which was only evident in *H. ovalis*. The results of the laboratory whole plant experiments, conducted under Sydney summer water temperature conditions, reflect those observed in the summer *in situ* experiments rather than those observed in winter *in situ*; suggesting laboratory experiments can guide what the field response could be, given temperature is an important consideration. $\Delta F/F_m$' appeared a more reliable indicator than that achieved with the chlorophyll *a* pigment analyses. These results suggest that assessments of seagrass health in laboratory experiments can in some cases be representative of that observed *in situ* when similar experimental conditions are maintained. However, large differences in the percent TPH recovered between *in situ* and laboratory experiments suggests microbial activity and sediments played a substantial role in the partitioning of oils in these experiments. This requires further investigation.

The findings of this study are that non-dispersed oil, in general, leads to less photosynthetic stress to *Zostera capricorni* and *Halophila ovalis* compared with the addition of a chemical dispersant. When the addition of a chemical dispersant is deemed necessary to protect other resources in the area, the seagrass may still recover depending on the dispersant used.

Introduction

The effects on subtidal seagrass from the application of dispersants to oil spills remain unclear (AMSA 2008). Whether to apply a chemical dispersant to an oil spill is usually a "trade-off" between the relative importance of subtidal resources and shoreline habitats. Dispersant application is often sought when sensitive shoreline resources (e.g. mangrove habitats, nesting bird colonies) are at a clear risk of oil contamination if the stranded oil were to be simply left to degrade and weather naturally. Chemically dispersing the oil into the water column may therefore be justified to decrease the risk of the oil coming closer to shore and to reduce the oil's overall persistence in the environment. In dispersing the oil spill, however, the hydrocarbon concentration in the water column is greatly increased and concurrently increases the potential risk to subtidal organisms, like seagrasses (NRC 2005; AMSA 2008).

Seagrass meadows are extremely productive environments (Hemminga & Duarte 2000; Mateo *et al.* 2006). They uptake nutrients from surrounding waters, act to stabilise the sediment and provide habitat and shelter for many species including those of commercial and recreational importance (Kirkman 1997; Hemminga & Duarte 2000). Declines in seagrass habitats have been reported worldwide as a result of natural events and human-induced stress (Larkum & West 1990; Kirkman 1997; Short & Wylie –Echevaria 2000; Green & Short 2003). Any controllable anthropogenic pressures need to be prevented or minimised where possible to reduce further degradation of these critical habitats.

Subtidal seagrass inhabit areas where oil spills commonly occur, in nearshore and inshore environments. The loss of 270 tonnes of heavy fuel oil from the *Pacific Adventurer* near the coast of Cape Moreton (QLD) in 2009; the breaching of the hull of the *Global Peace* (2006) in Port Curtis, Gladstone Harbour (QLD); and the spill of 95 tonnes of light crude oil from the *World Encouragement* in Botany Bay (1979) all occurred within close proximity to subtidal seagrass meadows. In the case of Botany Bay and Cape Moreton, at

least part of those seagrass meadows were, or now are, encompassed within RAMSAR wetlands of international significance (DEWHA 2010), highlighting both the extreme importance of the seagrass and the risk posed to these areas.

The body of evidence to suggest that seagrasses are impacted by oil is largely derived from when there has been a direct contact with the above ground biomass of the plant, the blades (Jacobs 1988). Even so, unless the oil is retained within the seagrass meadow for a sustained duration most studies report no long-term impacts to the meadow (Zieman *et al.* 1984; Jacobs 1988). Oil by itself affects seagrasses through the adsorption of the water accommodated fraction (WAF) which leads to a reduction in tolerance to other stress factors (Zieman *et al.* 1984). Smothering, fouling and asphyxiation are some of the effects that have been documented from oil contamination (Blumer 1971; Cintron *et al.* 1981). Seagrass blades have become bleached, blackened, yellowed and detached from the plant following direct oil contamination (Chan 1973; den Hartog & Jacobs 1980; Jackson *et al.* 1989; Dean *et al.* 1998;) whilst other effects from direct contact include a decrease in the density of shoots and flowering shoots (Chan 1973; den Hartog & Jacobs 1980; Dean *et al.* 1998). Zieman *et al.* (1984) suggests that in most cases the system has the ability for recovery however; damage to the rhizome-sediment structure may result in irreversible damage as the sediment stability becomes compromised. Subtidal seagrass can still be subjected to, and impacted by, direct contact with oil that has not been chemically dispersed. For example, subtidal beds of *Thalassia* and associated fauna were decimated following a crude oil spill in Puerto Rico (1973) (Naduau & Berquist 1977). Strong weather conditions caused the entrainment of oil into the subtidal seagrass beds. Incidentally, the crude oil was considered to be of low toxicity, yet the impacts to the seagrass were so severe that even the rhizome layer was affected.

Seagrasses have been shown to absorb more aliphatic and aromatic oil fractions when the oil is dispersed, therefore increasing the toxicity (den Hartog 1984). Dispersants are thought to affect the waxy cuticle of the seagrass blade and, in doing so, to increase the

penetrability of the dispersed oil to the photosynthetic organs, particularly the thylakoid membrane (Howard *et al.* 1989; Wolfe *et al.* 1998). Dispersed oil is also more susceptible than non-dispersed oil to microbial breakdown, which can lead to a greater oxygen demand by the microbes (Fingas 2001; NRC 2005). A reduction in the oxygen in the seagrass community may impact on the seagrass system (Zieman *et al.* 1984) as seagrasses have a high respiratory demand to support their large non-photosynthetic underground biomass.

Research outcomes from petrochemical exposure to seagrass range from dispersed oil posing a greater threat than non-dispersed oil; dispersed oil posing less of a threat than non-dispersed oil; and that neither oil nor dispersed oil negatively impact seagrass. This disparity amongst previous research findings may simply be an artifact related to the experimental differences between the studies relating to different methodologies, different exposure regimes, different species investigated, different petrochemicals exposed and different temperature conditions of the experiments. Morphological variety in seagrass is vast and species resilience to petrochemical impacts is likely to reflect this. Thorhaug *et al.* (1986) showed clear differences in the response of different species of tropical seagrass to petrochemicals but to date, other research has largely been conducted on single species (e.g. Baca & Getter 1984; Hatcher & Larkum 1984; Ralph & Burchett 1998; Macinnis & Ralph 2003). Different species of seagrass clearly respond differently to stressors, including that imposed by petrochemical pollution. Most studies, however, do not incorporate this into their research design likely due to the increased logistical effort of conducting multi-species analyses, specifically with aquatic macrophytes (Kuster & Altenburger 2007).

There is also disparity between results obtained from real spill events, *in situ* experiments and laboratory experiments particularly with the effects to subtidal seagrass (e.g. Macinnis & Ralph 2003). Field assessments are logistically intensive in time, cost and effort and as such fewer field assessments can be conducted compared with laboratory experiments. Laboratory experiments have been shown to overestimate the effects of real spills and *in situ* experiments, and many environmental variables are difficult to replicate in the

laboratory such as light attenuation (Clark & Noles 1994; Hemminga & Duarte 2000; Macinnis & Ralph 2003). The integration of both *in situ* and laboratory experiments may help in better understanding the effects of petrochemicals on seagrass and the disparity in research findings.

Finally, most studies assessing the effects of oil and dispersed oil on seagrass have focused on crude oil (e.g. Hatcher & Larkum 1984; Thorhaug *et al.* 1986; Baca *et al.* 1996; Ralph & Burchett 1998; Macinnis & Ralph 2003). Considering the recent spate of fuel oil (rather than crude oil) spill incidents in Australian waters (e.g. *Pacific Adventurer* 2009, *Global Peace* 2006), an understanding of the effects of these oils to subtidal seagrass is required if mitigation procedures are to be successful.

Aims

The major aims of this project were to:
1) determine the extent to which subtidal seagrass is affected by non-dispersed and dispersed crude and IFO-380 oil and at what concentrations;
2) determine the relative effects of different temperate species of seagrass to oil and dispersed oil; and
3) determine whether *in situ* experiments can be replicated in laboratory experiments.

Methods

Field Sites and Species

Collection of seagrass (and *in situ* experiments) were performed under a New South Wales Department of Primary Industry Scientific Research Permit (Permit numbers: P06-09/0010).

Two field sites were used for the *in situ* component of the study. The primary field site was Bonna Point, along the eastern shores of Botany Bay, New South Wales, Australia. Bonna Point was also the site for seagrass collection for the laboratory experiments. A smaller subset of experiments was also conducted in Corio Bay, along the south-western shores of Port Phillip Bay, Victoria, Australia. Only two treatments (non-dispersed and dispersed Tapis crude oil) were conducted in this location.

The *in situ* component comprised the assessment of *Z. capricorni* in summer and winter in Botany Bay; and *Z. muelleri* in Corio Bay in summer only. The laboratory experiments entailed the analysis of *Z. capricorni* and *Halophila ovalis* under summer conditions.

Exposure Regime

Seagrass were exposed to the water accommodated fraction (WAF) of oil, dispersed oil and dispersant alone, *in situ* using specially designed mesocosms, and in the laboratory using glass aquaria. Mesocosms and tanks were both approximately 12 L in volume. After a ten-hour exposure period, *in situ* mesocosms were removed allowing replenishment of 'fresh seawater'; whilst in the laboratory experiments, the aquaria were drained, and refilled with 'fresh' seawater. The seagrass were then further monitored over a four-day recovery period.

Photosynthetic assessment

Photosynthetic stress of the seagrass was monitored by chlorophyll *a* fluorescence using Pulse Amplitude Modulated (PAM) techniques and also via analyses of the chlorophyll *a* pigment concentrations. Chlorophyll *a* fluorescence, specifically, the effective quantum yield of Photosystem II ($\Delta F/F_m'$) was measured using a Diving-PAM and Mini-PAM (Walz, Germany) for *in situ* and laboratory measurements, respectively. The $\Delta F/F_m'$ provides information regarding the photosynthetic activity, thereby providing valuable information regarding the physiological health of the seagrass.

A 2 mm fibre optic was held in position close to the seagrass blade via specially designed leaf clips. The fibre optic extended outside of the exposure chamber (mesocosm/ aquaria) enabling remote PAM measurements to be taken without disturbing the experiment. Photosynthetic activity ($\Delta F/ F_{m'}$) was monitored every two hours during the exposure day, followed with once daily $\Delta F/ F_{m'}$ measurements for the next four days, the recovery period. Leaf samples were collected for the determination of chlorophyll *a* pigment concentrations at the end of the exposure day and also at the conclusion of the recovery period.

Water Accommodated Fraction

The water accommodated fraction (WAF) was prepared similar to Singer *et al.* (2000) and Macinnis and Ralph (2003). To produce a 1.00 % w/v solution, 50 g of oil was added to 5 litres of seawater in Erlenmeyer flasks, and stirred for 24 hours on magnetic stirrers. For the dispersed oil treatments, 5 g of dispersant was added and the water stirred for a further 10 minutes. The dispersant alone treatments were created by adding 5 g of dispersant to 5 litres of seawater and, consistent with the other treatments, stirred for 24 hours. All treatments were allowed to settle for one hour following the stirring stage. The WAF was siphoned into amber glass bottles stored at 4° C in darkness and used within two days (Singer *et al.* 2000). Petrochemical treatments, less than and including the 1.00 % WAF were created using the 1.00 % WAF with an appropriate amount of seawater for dilution. A 2.00 % WAF was created under a different loading regime, whereby 100 g of oil was added to 5 L of seawater.

Semi-quantitative analysis of the WAF (UVF) was performed prior to exposure, and at the end of the exposure period to assess the percentage total petroleum hydrocarbon (TPH) remaining in the water column for each experiment. Quantitative analysis (Gas Chromatography techniques) of the 1.00 % w/v WAF (pre-exposure) was also undertaken to provide details of the actual composition of each treatment. These quantitative analyses were performed by a commercial laboratory, Sydney Environmental and Soil Analysis

Laboratory (SESL) (NATA accreditation number 2901). These results are provided in the Appendix.

Petrochemical Treatments

The number and concentrations of the petrochemical treatments differed between experiments. More treatments and higher concentrations could be conducted under laboratory conditions than what could be conducted in the field. Petrochemical treatments included Tapis crude oil and IFO-380 oil, non-dispersed and dispersed, and dispersant alone treatments. Dispersants used were Corexit 9527, Ardrox 6120, Slickgone LTSW and Corexit 9500. Concentrations ranged up to 0.40 % WAF in the field and up to 2.00 % WAF in the laboratory experiments.

Results/ Discussion

All chlorophyll *a* fluorescence data ($\Delta F/F_m'$) from the field and laboratory experiments have been synthesised into Tables 1 and 2 for summary purposes and to enable a comparison of the results. To differentiate between the levels of impact, the magnitude of decline in $\Delta F/F_m'$ from the control has been classified as "Low" (< 0.15), "Medium" (0.15 – 0.30) and "High" (> 0.30). A "High" level effect (> 0.30 units $\Delta F/F_m'$) represents a reduction of greater than 50 % of the photosynthetic efficiency of the seagrass. The classification does not take into account where the seagrass recovered from a photosynthetic impact from the petrochemical treatment but where recovery, or lack of, occurred, it is highlighted in the text. The timing of impact details any sampling time within the exposure and recovery periods (where applicable) when a treatment significantly decreased in $\Delta F/F_m'$ from the control. The range of concentrations that caused these significant decreases in $\Delta F/F_m'$ is also included and is derived from the total petroleum hydrocarbon (TPH) concentration pre–exposure as detected by Gas-Chromatography Mass

Spectrometry analyses (Appendix). The 2.00 % water accommodated fraction (WAF) samples are represented as being greater than (>) the TPH of the 1.00 % WAF treatment. As the 2.00 % WAF treatment was created using a different loading regime, the TPH concentration cannot be calculated from the 1.00 % WAF treatment (Singer *et al.* 2000). Stimulation of growth (hormetic responses) occurred in most treatments and as these did not result in a negative impact during the experiment they have simply been marked with an *. For example, "No negative impact *" denotes that the $\Delta F/F_m'$ of the seagrass may have increased above the control at some sampling time from at least one concentration, at no time did any of the samples exhibit a decrease in $\Delta F/F_m'$ from the control.

Field Assessment

Treatments:

- *Tapis crude oil: Oil alone; Oil + Corexit 9527; Corexit 9527 alone*
- *IFO-380 oil: Oil alone; Oil + Slickgone LTSW; Slickgone LTSW alone*
- *Concentrations: 0.00, 0.05, 0.10, 0.20, 0.40 % WAF*

Neither the oil, dispersed oil or dispersant alone treatments resulted in a high level impact to the seagrass in summer or winter (Tables 1 & 2). No treatment led to an impact of greater than 0.2 units $\Delta F/ F_m'$ from the control. The initial $\Delta F/ F_m'$ was greater than 0.6 units in all samples. This means that no treatment exhibited a response greater than 30 % inhibition, with most showing a maximum response of far less than 20 % (0.1 units of photosynthetic activity below the control).

The magnitude of decrease in $\Delta F/ F_m'$ of *Z. capricorni* (Botany Bay) in the dispersed oil treatments (crude and IFO-380) was greater than the decrease in the oil alone or dispersant alone treatments (Tables 1 & 2), but this was only marginally so (within 0.1 units $\Delta F/ F_m'$). In some petrochemical treatments, the $\Delta F/ F_m'$ of the seagrass was actually enhanced. The results also showed that where negative responses were detected in the different

petrochemical treatments, they were only in the highest WAF concentrations, mostly in the 0.40 % WAF concentration, and in some experiments also in the 0.20 % WAF concentration. Most importantly, the seagrass showed complete recovery following any negative effect.

There was some evidence of seasonal variation between the summer and winter results for *Z. capricorni* but again, due to the lack of any great decrease in $\Delta F/ F_m'$, this was only minor (Tables 1 and 2). The magnitude of decrease in $\Delta F/ F_m'$ in the winter experiments was often greater than that observed in the summer experiments. This was true for the crude alone, IFO-380 alone, IFO-380 dispersed with Slickgone LTSW, and the Corexit 9527 alone treatments. However, these differences were generally within 0.1 units $\Delta F/ F_m'$ of their summer counterparts. The small seasonal variation is unlikely to affect oil spill decision making, but is worth consideration for further investigation.
Zostera muelleri in Corio Bay (Victoria) was not negatively impacted by the petrochemical treatments (Table 1). The seagrass actually displayed a significant increase in $\Delta F/ F_m'$ in most of the concentrations for both non-dispersed and dispersed crude oil. The chlorophyll *a* pigment concentration of the seagrass exposed to the crude alone treatment also showed an increase, but not until 96 hours following the initial exposure. The light intensities and temperatures subjected to *Z. muelleri* in Corio Bay, were likely to have been far greater than those experienced by *Z. capricorni* in the deeper waters of the Botany Bay experiments and are considered to have played a major role in this stimulation of growth. This is interesting, but should not influence the spill response decision.

Total petroleum hydrocarbon concentrations decreased to below detection limits by the end of the exposure period in all non-dispersed oil (crude and IFO-380) treatments. Only the greatest WAF treatments (0.20, 0.40 % WAF) of the dispersed crude and IFO-380 treatments displayed any measurable concentrations at the end of the exposure period, with generally no greater than 30 % remaining. There were slightly greater concentrations of both dispersed oils recovered in winter than in the summer.

Laboratory Assessment

Treatments:

- *Tapis crude oil: Oil alone; Oil + Corexit 9527; Oil + Ardrox 6120; Corexit 9527 alone; Ardrox 6120 alone*
- *IFO-380 oil: Oil alone; Oil + Slickgone LTSW; Oil + Corexit 9500; Slickgone LTSW alone; Corexit 9500 alone*
- *Concentrations: 0.00, 0.20, 0.40, 1.00, 2.00 % WAF*

Crude oil: non-dispersed, dispersed, dispersants alone

The crude alone treatment did not negatively impact *Z. capricorni* or *H. ovalis* (Table 1). This was supported by both the assessment of the chlorophyll *a* fluorescence data ($\Delta F/F_m'$) and the chlorophyll pigment analyses for both species. Considering that laboratory experiments often exaggerate the effects of oil and yet even the highest concentrations (2.00 % WAF) of the crude oil did not evoke a significant response from either species is an important finding for management.

The concern with dispersing an oil slick and the associated increased hydrocarbon content in the water column was somewhat supported by the results of these experiments (Table 1). The toxicity of the dispersed crude oil to *Z. capricorni* was specifically related to the actual dispersant used and the petrochemical loading in the water column. Whilst, both Corexit 9527 dispersed and Ardrox 6120 dispersed crude oil treatments produced negative impacts to *Z. capricorni*, the Ardrox 6120 dispersed treatment had a more sustained negative impact to the seagrass. For *H. ovalis*, $\Delta F/F_m'$ impacts were only detected within the first four hours, followed by full recovery, when exposed to either of the dispersed crude treatments. These laboratory experiments suggest that dispersed crude oil is more toxic to *Z. capricorni* and *H. ovalis* than non-dispersed Tapis crude oil; and that Ardrox 6120 dispersed crude oil is slightly more toxic than Corexit 9527 dispersed crude oil.

The dispersant alone treatments, unlike most of the impacts from the dispersed oil treatments, resulted in significant negative impacts to the seagrass even from the lower concentrations (0.20 and 0.40 % WAF) (Table 1). Both species showed a significant impact from the Ardrox 6120 alone treatments, far more than the Corexit 9527 alone treatments. *Zostera capricorni* was impacted greater than *H. ovalis* from the dispersant alone treatments as no significant differences were detected in *H. ovalis* with the Corexit 9527 exposure. In the case of Corexit 9527 exposure to *Z. capricorni*, the impact was far greater than the dispersant-oil combination. The results from this study suggest that spill response managers need to exercise care not to over apply dispersants to disperse a slick, as in some cases the dispersant alone treatment was actually more toxic than the oil-dispersant combination as in the case of the Corexit 9527 and *Z. capricorni*.

IFO-380 oil: non-dispersed, dispersed, dispersant alone

Zostera capricorni showed no impacts from the IFO-380 oil alone treatment (Table 2); however, H. ovalis exhibited a high level (> 30 % decline in $\Delta F/F_m'$) although short-lived (up to six hours) impact (Table 2). Where a response was detected in the dispersed IFO-380 oil treatments it was generally far greater than that which occurred from the non–dispersed IFO-380 oil.

The effects of the two dispersed IFO-380 oil treatments varied between treatments and also between species (Table 2). *Zostera capricorni* was impacted only by the Corexit 9500 dispersed IFO-380 oil treatment whereas *H. ovalis* was impacted by both dispersed IFO-380 oil treatments. There were also differences in the timing of the response of the seagrass. The effects to *Z. capricorni* from the Corexit 9500 treatment occurred most notably following the removal of the chambers, whereas to *H. ovalis* this effect occurred almost immediately. The high level impact to *H. ovalis* (Table 2) from the Slickgone LTSW dispersed IFO-380 oil over the exposure period was clearly different to the response of *Z. capricorni* which was not impacted negatively by the treatment (Table 2).

The dispersant alone treatments also resulted in differences between treatments within the one species and differences between species response from the same treatment (Table 2). *Halophila ovalis* was impacted more severely than *Z. capricorni* when exposed to the same dispersant treatment. The Slickgone LTSW alone treatment caused no detectable impact to *Z. capricorni* and a very short-lived impact to *H. ovalis* (eight hours exposure only). The Corexit 9500 alone treatment led to high level (> 30 % reduction in $\Delta F/F_m'$) impacts to both species. For *Z. capricorni* this level of impact was only detectable during two of the recovery days (24 and 48 hours). *Halophila ovalis*, however, exhibited this level of impact throughout most of the exposure and recovery days.

Most treatments displayed a high percentage recovery of total petroleum hydrocarbons suggesting minimal breakdown in these laboratory experiments over the exposure period. However, where a greater loss of TPH was evident in some treatments it appeared to correlate with a greater photosynthetic impact to the seagrass. The Tapis dispersed with Ardrox 6120 treatment showed a greater loss of TPH and resulted in greater photosynthetic stress to both species than the Corexit 9527 dispersed treatment. Similarly, both dispersed IFO-380 oil treatments also showed substantial loss of TPH over the exposure period which was also supported by an increased photosynthetic impact to both seagrass species. Dispersant alone treatments were also analysed with this semi-quantitative method with the results suggesting minimal loss of TPH over the exposure period. The results of these dispersant alone treatments should be treated with caution, however, as they may reflect backscattering of the UV light within the sample rather than any hydrocarbons. Further research into the use of oil-in-water fluorometers for treatments containing dispersants would increase the confidence of results with samples containing dispersants.

Conclusions/ Recommendations

There were clear differences in the response of the three species analysed in this study when exposed to the petrochemicals; this supports similar findings by Thorhaug *et al.*

(1984). In the field experiments, the stimulation of growth in *Z. muelleri* in Corio Bay (VIC) is suggested to be partly due to the difference in the light attenuation and water temperature compared to Botany Bay (NSW). In the laboratory assessment, the morphological differences between *Halophila ovalis* compared with *Zostera capricorni* were considered to have played a significant role in the species resilience to the petrochemicals. The shape of the blade and the positioning of the seagrass in the water column (paddle-shaped blade in *H. ovalis* and close to the sediment; strap-like blade in *Z. capricorni* and higher in the water column) are two possible morphological differences that may have led to these differences.

Seasonal variation in the response of *Z. capricorni* to these petrochemicals appears slight, but likely, and supports other research findings on seasonal variation in plant sensitivity from oil pollution (eg. Pezeshki *et al.* 2000) and to other toxicants (Brun *et al.* 2002). Future research into the effects of water temperature into seagrass response to petrochemicals may shed more light on this factor, and also the enhancement of growth in *Z. muelleri*.

Table 3 Summary table of magnitude of stress ($\Delta F/F_m'$), timing of impacts and effective concentrations in *Z. muelleri, Z. capricorni* and *H. ovalis* from exposure to the crude oil treatments (non-dispersed, dispersed and dispersant alone). * not significantly different to control. na treatment was not performed under those conditions. See text for further explanation.

	Field Experiments			*Laboratory Experiments*	
	Z. muelleri	*Z. capricorni*	*Z. capricorni*	*Z. capricorni*	*H. ovalis*
Treatment	Summer	Summer	Winter	'Summer'	'Summer'
Crude	No negative impact *	No negative impact *	**Low 6-8, 48 h 2.4-4.8 mg L^{-1}**	No negative impact	No negative impact
Crude + C9527	No negative impact *	**Medium 4, 24 h 20-40 mg L^{-1}**	**Medium 4 h 40 mg L^{-1}**	**Low 6-8 h >101 mg L^{-1}**	**Medium 2h >101 mg L^{-1}**
Crude + Ardrox 6120	Na	Na	Na	**Medium 8-72 h >105 mg L^{-1}**	**Medium 2h >105 mg L^{-1}**
C9527	Na	No negative impact	**Medium 4 h 64 >128 mg L^{-1}**	**Medium 2, 6-8, 24-96 h 32 >317 mg L^{-1}**	**Medium 48-96 h >317 mg L^{-1}**
Ardrox 6120	Na	Na	na	**Medium 4-48, 96 h 92 >230 mg L^{-1}**	**High 2-4, 24-96 h 92 >230 mg L^{-1}**

Table 4 Summary table of magnitude of stress ($\Delta F/F_m'$), timing of impacts and effective concentrations in *Z. capricorni* and *H. ovalis* from exposure to the IFO-380 treatments (non-dispersed, dispersed and dispersant alone). * not significantly different to control. na treatment was not performed under those conditions. See text for further explanation.

	Field Experiments		*Laboratory Experiments*	
	Z. capricorni	*Z. capricorni*	*Z. capricorni*	*H. ovalis*
Treatment	Summer	Winter	'Summer'	'Summer'
IFO-380	No negative impact *	**Low 2-4, 48 h 1 mg L^{-1}**	No negative impact *	**Medium 2-4 h 1 >3 mg L^{-1}**
IFO-380 + Slickgone LTSW	No negative impact *	**Medium 2-4 h 80 mg L^{-1}**	No negative impact	**High 4-8, 24, 96 h 80 >200 mg L^{-1}**
IFO-380 + C9500	Na	na	**Medium 6-48 h 200 >500 mg L^{-1}**	**Medium 2-6, 10-24 h 200 >500 mg L^{-1}**
Slickgone LTSW	No negative impact *	No negative impact *	No negative impact	**Medium 8 h >150 mg L^{-1}**
C9500	Na	na	**High 24-48 h 67 >167 mg L^{-1}**	**High 2-48, 96 h >167 mg L^{-1}**

Clear differences were detected in this study regarding the amount of TPH recovered between the field and laboratory experiments. In the *field* experiments, there was minimal, if any, TPH recovered following the exposure period in most treatments which contrasted with the relatively high levels of TPH recovered following the same duration of exposure in the laboratory experiments. As sediments and microbial activity play a major role in the breakdown of petrochemicals (Leahy & Colwell 1990; Fingas 2001), a reduction of TPH found in these in laboratory experiments (Clark & Noles 1994) has led several authors to suggest that an increase in photosynthetic stress to organisms in laboratory experiments may be derived from a reduced rate of petrochemical breakdown by these microorganisms (eg. Macinnis & Ralph 2003). In the whole plant laboratory experiments in the current study, there would have been fewer microbes due to the filtered seawater and reduced amount of sediment when compared with the *in situ* experiments. Considering these factors, it is likely that the smaller loss of TPH in the laboratory experiments compared to the *in situ* experiments is due to the reduced microbial activity and sediments in the laboratory experiments.

Major findings from this study and recommendations for oil spill management:

- Even the highest concentrations (2.00 % WAF) of the crude oil did not evoke a significant response from either *Z. capricorni* or *H. ovalis*.
- In most cases the addition of dispersant to either Tapis crude or IFO-380 oil increased the stress response from the seagrass. It appears that it would be preferable, where possible, to not disperse either of these oils over regions of subtidal meadows of *Z. capricorni* or *H. ovalis*.
- Certain dispersants were more toxic to specific species of seagrass.
- Corexit 9527 dispersed crude oil appeared slightly less toxic than Ardrox 6120 dispersed crude oil to both *Z. capricorni* and *H. ovalis* and would therefore be considered slightly more favourable.
- Slickgone LTSW dispersed IFO-380 oil (and Slickgone LTSW alone) was less toxic to *Z. capricorni* than the respective Corexit 9500 *treatments and would be a more suitable dispersant when this species is concerned.

- *Halophila ovalis* may be severely impacted by both dispersants and recommendations are to not disperse with either Slickgone LTSW or Corexit 9500. This species, however, is considered to recover rapidly from stress events, therefore where the species occurs in the same meadow as *Z. capricorni*, it may be more beneficial to focus response efforts on *Z. capricorni*.
- Most dispersant alone treatments caused photosynthetic stress to the seagrass, and in some cases this was greater than the dispersed oil and the oil alone treatments. It is strongly recommended that care is exercised not to over apply dispersants to an oil slick in the vicinity of either *Z. capriconi* or *H. ovalis*.

In summary, few treatments led to severe and, or, prolonged impacts to the seagrass investigated in this study. Where a photosynthetic impact to the seagrass was detected this was followed by full recovery in all field experiments and in the majority of the laboratory experiments. This finding implies that these species are quite resilient to the petrochemicals used under the experimental conditions of the study.

As the dispersed treatments did in most cases result in a photosynthetic impact to some degree, the results further suggest that when possible, it is better to not disperse over an area of subtidal seagrass when either Tapis crude oil or IFO-380 is spilt. However, when the addition of chemical dispersant is deemed appropriate to protect other resources within the area the seagrass may still recover depending on the dispersant used.

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Appendix: Chemical analysis of the water accommodated fraction

The total petroleum hydrocarbon (TPH) concentration (Σ C_6 to C_{36}) for the 1.00 % crude alone water accommodated fraction (WAF), pre-exposure, was 12 mg L^{-1}(Fig. 1). Compared to the other treatments, this TPH was quite low. However, it was comprised of approximately 80 % of highly volatile, light weight hydrocarbons in the C_6 to C_9 range, by far the highest percentage composition of C_6 to C_9 hydrocarbons in any of the treatments; crude or IFO-380. The addition of the dispersants to the crude oil increased the TPH in the WAF by almost ten-fold. The Corexit 9527 and the Ardrox 6120 dispersed oil treatments showed very similar TPH, (101 and 105 mg L^{-1}, respectively) but did differ somewhat in their composition. The TPH within the dispersant Corexit 9527 alone and Ardrox 6120 alone treatments was high, 317 and 230 mg L^{-1} respectively. The high TPH concentration in the dispersant alone treatments compared

with the non-dispersed and dispersed oil treatments is likely partially due to the one hour settling time of the treatments prior to siphoning; with a resurfacing of some of the oil and dispersed oil.

Toluene was the most abundant of the BTEX hydrocarbons within the non-dispersed and dispersed crude treatments (Fig. 2). Interestingly, the composition of the BTEX hydrocarbons, albeit slightly greater in the dispersed crude treatments, was very similar to the non-dispersed crude treatment. The dispersant alone treatments showed only minimal concentrations of the BTEX hydrocarbons.

The TPH within the IFO-380 WAF treatment was low compared to all other treatments 3 mg L^{-1} (Fig. 3). Both dispersed IFO-380 treatments increased the TPH within the WAF greatly, with the Corexit 9500 dispersed treatment increasing the amount by almost 200 times greater than the non-dispersed IFO-380 treatment (Fig. 3). The TPH within the Slickgone LTSW alone and Corexit 9500 alone treatments were 150 and 167 mg L^{-1} respectively (Fig. 3).

Low levels of BTEX hydrocarbons were detected in the IFO-380 oil treatments compared to the crude oil treatments (Fig. 4). BTEX hydrocarbons represented about 20 % of the total TPH of the IFO-380 alone WAF treatment with 82 % of the BTEX total made up of toluene and xylene (Fig. 4) The concentration of BTEX components within the water accommodated fraction decreased with the addition of the dispersants, but only slightly. The dispersant alone treatments were compromised of twice as much BTEX components than their respective dispersed IFO-380 treatments.

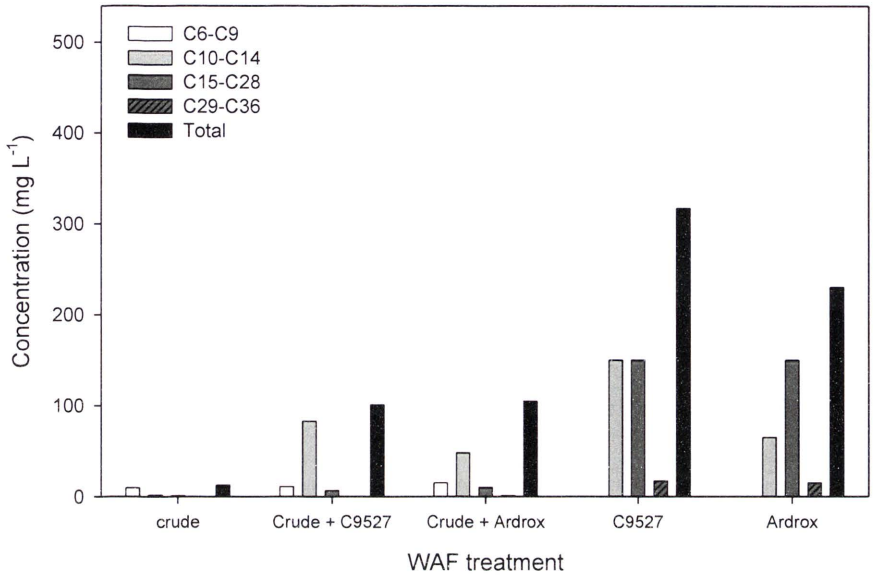

Figure 1: Carbon chain length fractionation per treatment and total petroleum hydrocarbon concentration (mg L^{-1}) within the crude, crude + Corexit 9527, Crude + Ardrox 6120, Corexit 9527 alone, Ardrox 6120 alone WAF treatments pre-exposure (n = 1).

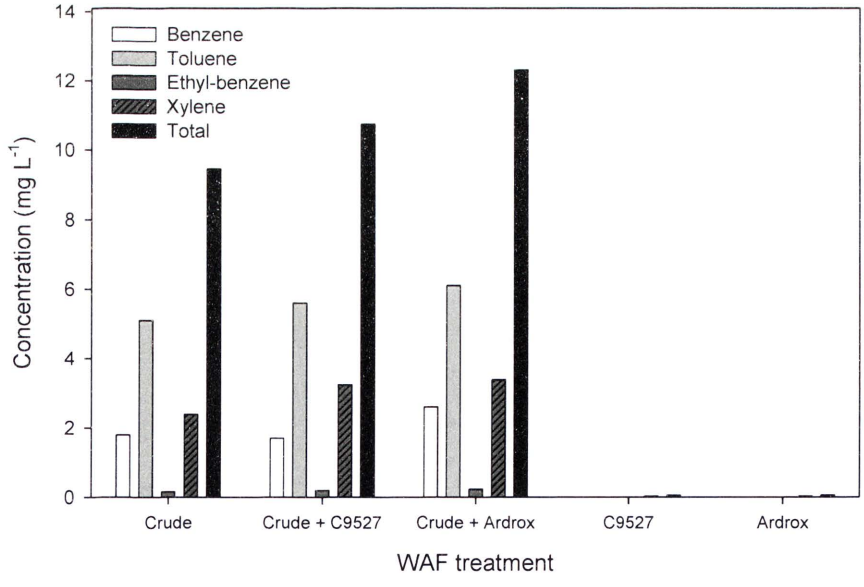

Figure 2: BTEX composition (mg L^{-1}) within the crude, crude + Corexit 9527, Crude + Ardrox 6120, Corexit 9527 alone, Ardrox 6120 alone WAF treatments pre-exposure (n =1).

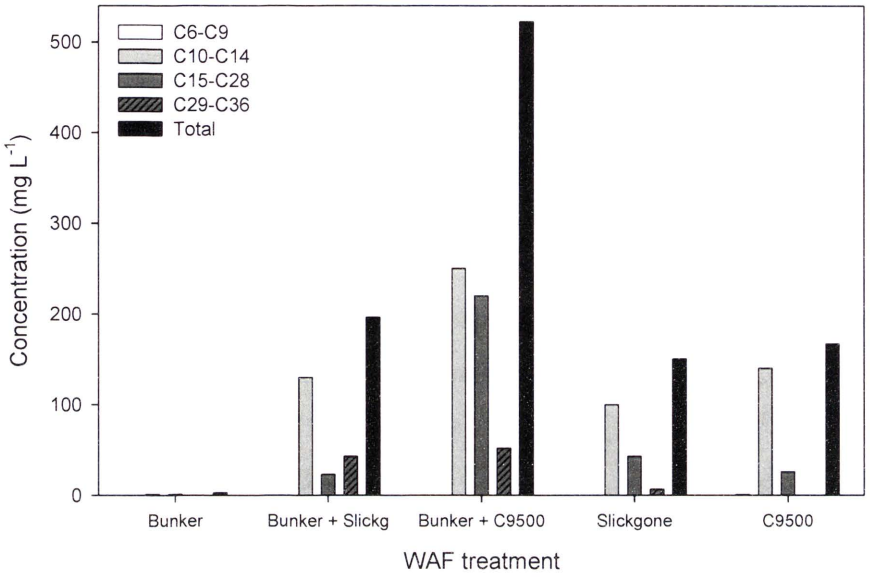

Figure 3: Carbon chain length fractionation per treatment and total petroleum hydrocarbon concentration (mg L^{-1}) within the IFO-380, IFO-380 + Slickgone LTSW, IFO-380 + Corexit 9500, Slickgone LTSW alone and Corexit 9500 alone WAF treatments pre-exposure (n = 1).

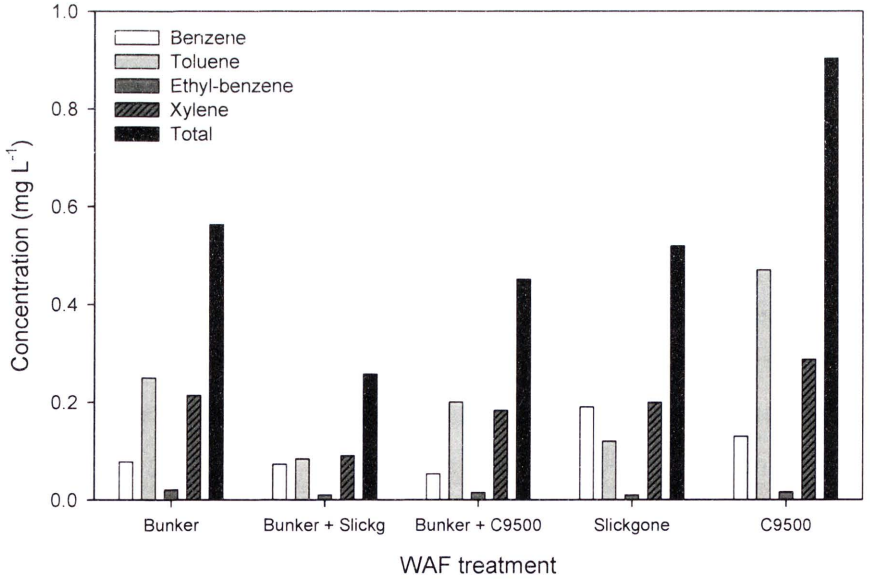

Figure 4: BTEX composition (mg L^{-1}) within the IFO-380, IFO-380 + Slickgone LTSW, IFO-380 + Corexit 9500, Slickgone LTSW alone and Corexit 9500 alone WAF treatments pre-exposure (n =1).

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